Bioreaction engineering for the kinetic resolution of racemic epoxides by epoxide hydrolase

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RIJKSUNIVERSITEIT GRONINGEN

Bioreaction Engineering for the Kinetic Resolution of Racemic Epoxides by Epoxide Hydrolase

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Chapter 1

General Introduction

The use of biocatalysts for the production of fine chemical and pharmaceutical intermediates is becoming increasingly common because of their ability to catalyse reactions with high stereo-, regio-, and chemo-selectivity. Biotransformation steps can either complement chemical steps or exist as alternative synthesis routes for a given compound, often with the advantage of reducing the number of reaction steps required (Cheetham, 1998). In some cases biocatalysis has provided new routes for the synthesis of high value compounds that cannot be produced by conventional chemo-catalysis. Synthesis of products with multiple chiral centres is one key area where the selectivity of biocatalysts can be exploited.

Biotransformations are generally recognised as being environmentally friendly compared to chemical processes since water is often the solvent of choice and reactions are catalysed at ambient temperatures and pressures. The high specificity of the reactions often also means that fewer side products are produced. Nevertheless, at the industrial scale, biotransformations must be economically competitive with chemical processes in terms of productivity and cost. It is precisely this lack of economic competitiveness which in some cases has led to the slow acceptance of bioprocesses as suitable alternatives to chemical processes. To be economically competitive a biotransformation must be operated at high volumetric productivity, which is defined as:

$$\text{volumetric productivity} = \frac{\text{amount of product produced}}{(\text{reaction time}) \cdot (\text{reaction volume})} \qquad (1.1)$$

Clearly, high productivity is achieved if the product is present in the reaction volume at a high concentration and if the time taken for reaction is low. It is also desirable to maximise the yield of the reaction to reduce the formation of waste by-products, especially when reactant costs and the cost of waste disposal are high. In addition, biocatalyst productivity (amount of product formed per amount of catalyst consumed) should be as high as possible since biocatalyst costs can be relatively important (Tischer and Kasche, 1999; Tramper, 1996). Good biocatalyst productivity requires high biocatalyst stability and effective recycle of the biocatalyst. Bioprocess development should be aimed at maximising these factors while satisfying key process requirements such as product (enantio)purity. Good integration between the biocatalytic step and downstream processing is also important in the overall optimisation of a bioprocess. In this respect, the use of high product concentrations is desirable to ease product recovery and purification. Reaction engineering for the production of optically pure epoxides by biocatalytic kinetic resolution, aimed at achieving high product and biocatalyst productivity, is the subject of this thesis.

Synthesis of optically pure epoxides

Optically pure drugs are increasingly being used since they are more target specific and have fewer side effects than racemic mixtures (Kloosterman, 1988). This is because different enantiomers of a compound can have different pharmacokinetic properties. The sale of single isomer drugs reached 32% of the total drug sales world wide in 2000 and further increases in market share are forecast (Stinson, 2001). This increase has resulted due to the requirement by the regulatory agencies for testing both enantiomers of a racemate in pharmacological studies during the drug approval process (Persidis, 1997), and also reflects the fact that drug companies are using chirality to extend the patent lives of block buster drugs by patenting single isomer versions of the racemates (Stinson, 2001). Epoxides are extremely useful chemical and pharmaceutical intermediates since the epoxide functional group can easily undergo ring opening, accepting a wide variety of nucleophiles (De Vries and Janssen, 2003). For example, epoxides are attractive intermediates in the production of β-blockers, insect pheromone (Kloosterman, 1988) and the herbicide (*S*)-indofan (Tanaka et al., 2002). The need to introduce chirality in the earliest steps of a synthetic route has made the production of chirally pure epoxides all the more important.

Chemocatalytic routes for the production of optically pure epoxides include epoxidation of allylic alcohols and conjugated alkenes, reactions developed respectively by Sharpless/Katsuki and Jacobsen (Jacobsen, 2000), which make use of transition metal complexes as catalysts (Archer, 1997). High enantiomeric purity and reasonable yields can be achieved with these methods, however, they can be used only for specific substrate structures and require the undesirable use of heavy metals. New metal containing salen complexes which catalyse the kinetic resolution of terminal epoxides to high enantiopurity have also been developed (Furrow et al., 1998; Tokunaga et al., 1997; Yoon and Jacobsen, 2003). The most promising biocatalytic routes for the production of enantiopure epoxides include stereospecific epoxidation of alkenes by monooxygenases (Archer, 1997; Schmid et al., 2001), and the resolution of racemic epoxides by epoxide hydrolases (Steinreiber, 2001). More recently, the scope of producing optically pure epoxides by enantioselective kinetic resolution of racemic halohydrins by direct ring closure mediated by halohydrin dehalogenases has been investigated (Lutje Spelberg et al., 2002b).

Asymmetric synthesis of epoxides by monooxygenases is attractive because the maximum theoretical yield of enantiopure epoxide is 100%. Monooxygenases are cofactor-dependent enzymes, and therefore whole cells are usually employed for easy cofactor regeneration (Duetz et al., 2001). Since oxygenases often have low specific activities, research efforts on this biosynthetic route have concentrated on the formation of recombinant *E. coli*

strains which express monoxygenases at high levels in order to achieve high productivity in preparative applications (Schmid, 2001).

Optically pure epoxides can also be obtained by kinetic resolution, whereby whole cells or enzymes degrade a racemic epoxide mixture stereoselectively. An inherent disadvantage of this method is that the maximum yield is limited to 50% since the unreacted enantiomer of the racemic mixture is the desired product. However, this route remains attractive since racemic mixtures are relatively cheap and since optically pure diols are also useful intermediates. Attempts to increase the yield of optically pure epoxide have made use of epoxide hydrolases with complementary enantiospecificities so that optically pure diols are obtained in high yields (92%) (Pedragosa-Moreau et al., 1993). In another example, acid catalysis was used to convert the remaining epoxide enantiomer to give enantiomerically pure diol in high yield (>90%) (Pedragosa-Moreau, 1997). To obtain enantiopure epoxide, chemical ring closure of the formed enantiomerically pure diol with retention of enantioconfiguration would be a necessary extra step. In the kinetic resolution of 4-isobutyl-α-methyl styrene oxide, Cleij et al. (1999) obtained enantiopure epoxide in high yield by recycling the produced unwanted diol to racemic epoxide by treatment with HBr/AcOH, followed by cyclization of the formed bromohydrin under basic conditions.

Epoxide hydrolase from *A. radiobacter* AD1

Epoxide hydrolases catalyse the addition of a water molecule to an epoxide (Fig. 1.1). Epoxide hydrolases are cofactor independent enzymes and are ubiquitous in nature, having been found in mammalian cells, bacteria, yeast, fungi, plants and insects (Archer, 1997). In mammalian cells, microsomal epoxide hydrolase activity is highest in organs such as the liver, kidney and lungs and the enzymes have the important function of catalysing the conversion of a broad array of xenobiotic substrates to more polar metabolites (Seidegard and De Pierre, 1983). This function is particularly important since the high reactivity of epoxides with nucleophiles makes them potent mutagens and carcinogens by modification of DNA by alkylation (Mlejnek and Kolman, 1999). Similarly epoxide hydrolases in bacteria can be part of a degradation route for xenobiotic compounds. Again, the toxicity of epoxides to cells in the degradation of chlorinated compounds has been attributed to the high reactivity of epoxides towards cellular nucleophiles such as proteins and nucleic acids (Oldenhuis et al., 1991).

The work described in this thesis concerns the use of an epoxide hydrolase of bacterial origin, originally isolated form the organism *Agrobacterium radiobacter* AD1 (van den Wijngaard et al., 1989) which can grow on and thus degrade epichlorohydrin to glycerol. The degradation is mediated by a haloalchohol dehalogenase and an epoxide hydrolase. The gene coding for the epoxide hydrolase has been cloned and brought to

overexpression in *E. coli*. This opened the possibility for its exploitation for preparative biocatalysis since typical enzyme production levels increased from 1-2 mg L^{-1} culture for the original organism to 200 mg L^{-1} culture (Rink et al., 1997).

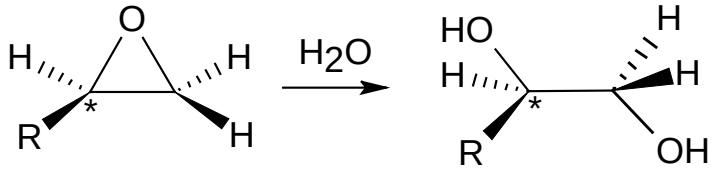

Figure 1.1. Reaction catalysed by epoxide hydrolase. The chiral centre is indicated with an asterix.

This enzyme has a molecular mass of 34 kDa and is monomeric in solution. The X-ray structure has been solved and shows that this enzyme belongs to the α/β hydrolase fold family of enzymes (Nardini et al., 1999). The catalytic triad residues are Asp107, His275, Asp246 and are located in a cavity between the core and cap domains of the enzyme molecule. The catalytic mechanism comprises two steps, the formation of an alkyl-enzyme intermediate by nucleophilic attack of a carboxylate oxygen of an aspartate residue on the least hindered carbon atom of the epoxide, followed by hydrolysis of the intermediate by a water molecule which is activated by deprotonation by a general base (His275) (Rink and Janssen, 1998) (Fig. 1.2). Two tyrosine residues, Tyr152 and Tyr215, which are found on the α-helical cap domain, point into the active site and serve as proton donors in the opening of the epoxide ring. Both tyrosine residues help stabilise the alkyl-enzyme intermediate by hydrogen bonding of the epoxide oxygen (Rink et al., 2000).

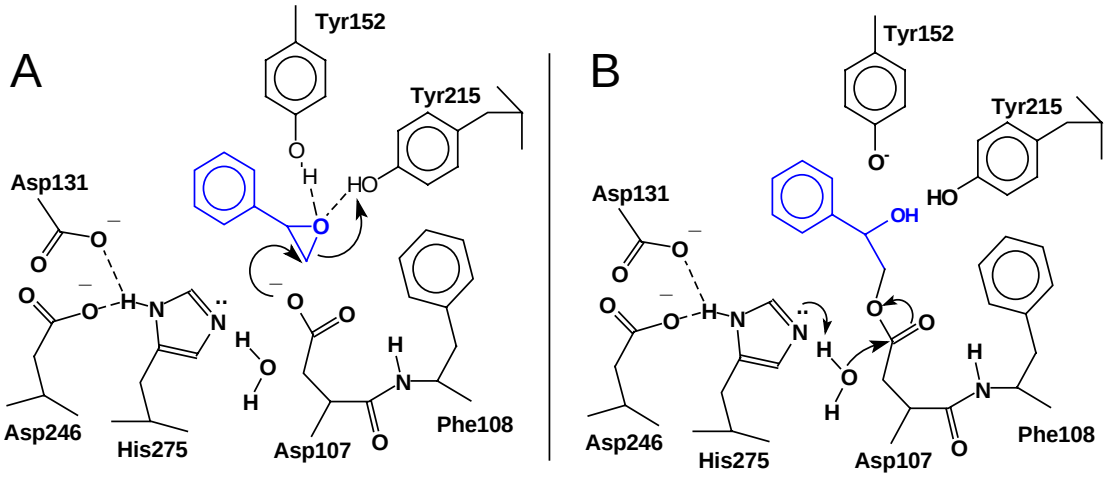

Figure 1.2. Reaction mechanism of the epoxide hydrolase from *A. radiobacter* AD1. (A) alkylation reaction and (B) hydrolysis of covalent intermediate.

The wild-type enzyme hydrolyses a broad range of terminal epoxides. The hydrolysis of (substituted) styrene epoxides such as styrene oxide and *p*-nitrostyrene oxide, proceeds with moderate to high enantioselectivity (Lutje Spelberg et al., 1998, 2002a) yielding the optically pure (*S*)- enantiomers of the epoxides. The hydrolysis proceeds with a high degree of regioselectivity favouring attack on the less sterically hindered carbon atom of the oxirane ring with retention of configuration. Mutation of one of the two active site tyrosines to phenylalanine results in active mutants which differ in conversion kinetics compared to the wild-type enzyme. The mutation affects primarily substrate binding and the alkylation half reaction (Rink et al., 1999). Overall, the single tyrosine mutants show a higher enantioselectivity to various epoxides than the wild-type enzyme and are therefore valuable for industrial biocatalytic application. The use of the Y215F mutant epoxide hydrolase is evaluated in *Chapter 5*.

Both the wild-type and active site mutants of this enzyme have a broad pH optimum between pH 6 and 10 (Rink et al., 2000) and the active site mutants were found to be more stable than the wild-type enzyme at higher pH. Further studies of operational stability of the enzymes are described in *Chapters 3* and *4*.

Characterisation of kinetic resolution reactions

The enantiopurity of a substrate or product is usually described in terms of enantiomeric excess (e.e.) which is calculated by,

$$\text{e.e.} = \frac{([R]-[S])}{([R]+[S])}, \tag{1.2}$$

where [S] and [R] are the concentrations of the enantiomers. Another parameter used to quantify the selectivity of a kinetic resolution is the enantiomeric ratio, E (Chen et al., 1982). The enantiomeric ratio is defined as the ratio between the specificity constants for the conversion of the two enantiomers ($(k_{cat}/K_m)_R/(k_{cat}/K_m)_S$) and is an intrinsic property of the enzyme. The E value of a conversion can be calculated by knowing the substrate enantiomeric excess (ee_S) achieved for a certain conversion (X) using the equation:

$$E = \frac{\ln\left[(1-X)(1-ee_S)\right]}{\ln\left[(1-X)(1+ee_S)\right]} \tag{1.3}$$

The relationship between e.e. and conversion is shown in Figure 1.3 for different values of E. The figure illustrates one of the advantages of kinetic resolution, namely that the desired e.e. can be achieved by setting the necessary conversion, even though this occurs at the expense of the yield of remaining enantiopure substrate. An enantiomeric ratio greater than 20 is a minimum prerequisite for industrial application (Sheldon, 1996).

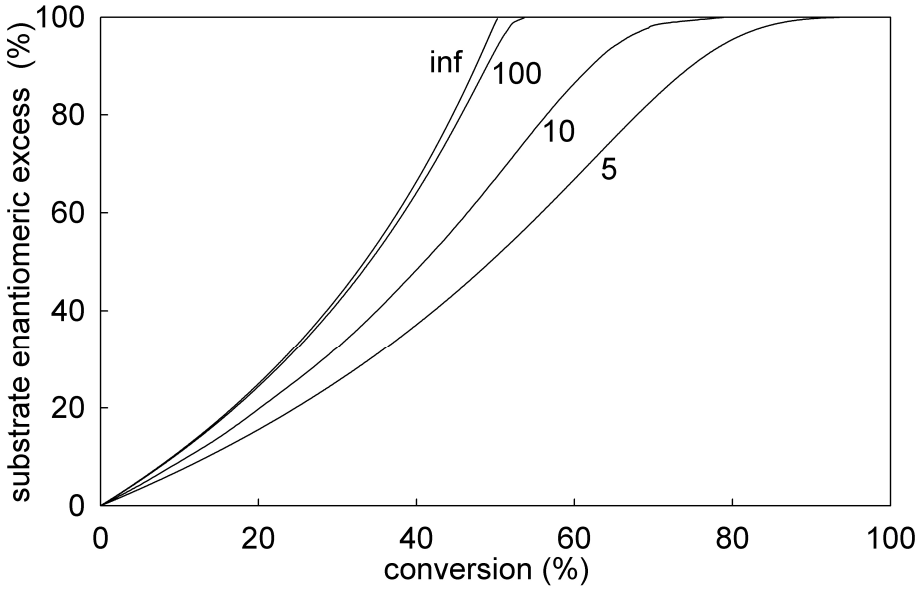

Figure 1.3. Change in substrate enantiomeric excess with conversion during kinetic resolution shown for different values of the enantiomeric ratio, E. The figure shows that the conversion required to achieve high enantiomeric excess of substrate increases for decreasing values of E. For industrial application, an E value greater than 20 is required (Sheldon, 1996).

In a few cases, resolution reactions follow sequential conversion kinetics, whereby one enantiomer is converted before another. This type of kinetics has been reported for the conversion of *tert*-butyloxirane by microsomal epoxide hydrolase (Watabe et al., 1981; Wistuba and Schurig, 1992). An important example of this type of conversion kinetics is the hydrolysis of styrene oxide by the wild-type epoxide hydrolase (Rink and Janssen, 1998) (Fig. 1.4, Table 1.1). This epoxide is used as a model substrate for much of the work described in this thesis. Sequential conversion is due to the much higher affinity of the (*R*)-enantiomer for the active site, so that the conversion of the (*R*)-enantiomer inhibits the conversion of the (*S*)-enantiomer. For this substrate the (*R*)-enantiomer is first converted with a k_{cat} of 3.8 s^{-1} and then the (*S*)-enantiomer is converted with a k_{cat} of 10.5 s^{-1} so that once the (*R*)-enantiomer is depleted the (*S*)-enantiomer is converted at a much faster rate. The implications of this type of complex kinetics for the operation of large scale resolution processes are that the end-point of the reaction has to be well controlled to maximise the yield of remaining pure substrate enantiomer. If the reaction is stopped before the ideal end-point then the enantiomeric excess may be too low, if the optimum end point is passed then the yield of pure enantiomer decreases quickly. For more classical conversion kinetics (Fig. 1.4), for example in the conversion of styrene oxide by the Y215F epoxide hydrolase mutant, these

considerations are still valid but less critical since the enantiomer which is hydrolysed last is usually converted at a lower rate.

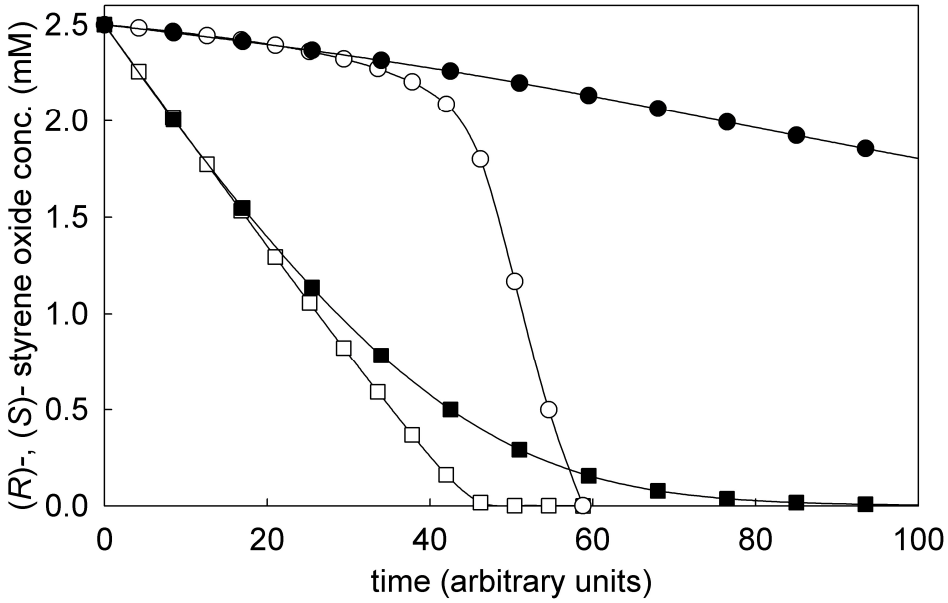

Figure 1.4. Simulated conversion curves for the kinetic resolution of styrene oxide by wild-type (□ (*R*)-enantiomer, ○ (*S*)-enantiomer) and Y215F mutant (■ (*R*)-enantiomer, ● (*S*)-enantiomer) epoxide hydrolase. The conversion profile for the wild-type enzyme is typical for sequential kinetics where the conversion of the remaining enantiomer follows the conversion of the first enantiomer. This effect is less dramatic for the conversion by the Y215F mutant enzyme since the second enantiomer is only slowly converted. Concentration profiles were obtained by equations describing competitive Michaelis-Menten kinetics of the type, $\frac{d[S]}{dt} = -\frac{k_{cat}^{S}[E][S]}{[S] + (\frac{[R]}{K_M^R} + 1)K_M^S} - k_c[S]$. Kinetic constants used are given in Table 1.1. Simulations were carried out using the software package Scientist™ (MicroMath Inc., Salt Lake City, UT).

Table 1.1. Steady state kinetic parameters of wild-type and mutant epoxide hydrolase for the enantiomers of styrene oxide (Rink et al., 2000)

	(*R*)-styrene oxide			(*S*)-styrene oxide		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
wild-type	3.8	0.0005	8000	10.5	0.021	500
Y215F	2.5	0.6	4.2	0.7	5	0.14

Bioprocess Design

Bioprocess design involves identifying key characteristics of the reactants, products and the biocatalyst which impose constraints on the bioprocess performance (Lilly, 1997). The productivity of kinetic resolutions of racemic epoxides is constrained by several factors. Many epoxides, such as epoxyoctane, p-nitrostyrene oxide and styrene oxide have a solubility of less than 5 g/L at 30 ^{o}C, limiting the productivity of reactions which can be carried out in aqueous solutions. The use of organic solvents to reduce this limitation is described below. In addition, epoxides tend to be unstable in water and undergo spontaneous chemical hydrolysis to their diols. A typical value for the first order rate constant for spontaneous hydrolysis is $1x10^{-5}$ s^{-1}. Since chemical hydrolysis occurs in a non-enantioselective manner, this side reaction results in a decrease in the yield of enantiomerically pure substrate and also a decrease in the enantiopurity of the formed diol. The calculated E-value of a resolution based on e.e. vs. conversion data (Fig. 1.3) will be lower than the intrinsic E-value for the enzyme if such a non-selective side reactions occurs. To minimise the loss of yield from spontaneous chemical hydrolysis, the rate of the enzymatic reaction has to be high relative to that of chemical hydrolysis (*Chapter 2*) and since chemical epoxide hydrolysis is favoured at extremes of pH, the operating pH should be selected appropriately. Diols, on the other hand, are stable in water and generally have a high aqueous solubility at 30 ^{o}C. In several different studies, however, inhibition of epoxide hydrolase activity by diols has been proposed as one of the causes for reduction in conversion rates during epoxide kinetic resolution reactions (Choi et al., 2000; Genzel et al., 2001a; Tang et al., 2001). None of these studies have explored the type and reversibility of this inhibition, and this is investigated in *Chapter 4*.

Multiphase systems

Water miscible organic solvents such as DMSO, acetone and ethanol are often added to reaction mixtures to increase the aqueous solubility of poorly water soluble reactants. At low solvent concentrations this strategy can be effective without adversely affecting enzyme activity and stability. At higher cosolvent concentrations, however, biocatalyst inhibition or inactivation may become prohibitive, thus limiting the maximum cosolvent concentration which may be used. A disadvantage of this approach is that the use of miscible cosolvents does not automatically simplify downstream recovery of the biocatalyst or product separation.

Alternatively, immiscible apolar organic solvents can be used to create a multiphase system where the organic solvent acts as a reservoir for the poorly water soluble substrate, allowing much higher substrate concentrations to be present in the reaction volume. In these biphasic systems, the substrate is transferred to the aqueous phase and is converted by

the enzyme contained therein. Multiphase systems can be used advantageously to facilitate downstream separation of substrates and products depending on partitioning of these species between the phases. Appropriate selection of the organic phase must take into account these partitioning effects so that inhibitory products can be removed and substrates can be fed at sub-inhibitory levels. Optimal partitioning can also minimise unwanted side reactions. Minimising unwanted epoxide chemical hydrolysis by keeping the concentration of epoxides low in the aqueous phase is a good example of this. Optimisation of solvent physical properties is also important with respect to downstream processing since separation of emulsified layers can be a problem on an industrial scale (Kloosterman, 1988). The advantages afforded by multiphase systems listed above, prompted us to investigate the use of a two-liquid-phase system for carrying out the kinetic resolution of epoxides at high substrate concentrations (*Chapter 2*).

Ideally, the use of organic solvents should be avoided since safety issues concerning solvent flammability and toxicity arise for bioprocess operation and formulation of the final product. This can be achieved if the substrate itself constitutes the separate phase. Both solid and liquid substrates have been used in this context (Straathof et al., 2002). Recently ionic liquids have emerged as potential replacements of organic solvents since they are non-flammable and non volatile, and are therefore less toxic to operators and the environment. The use of these liquids is reviewed by Kragl et al. (2002) and will not be described further here.

The advantages of using liquid-liquid systems to increase substrate concentrations can be offset by the inhibiting and inactivating effects that organic solvents can have on the biocatalyst. Toxic effects of solvents on the activity and stability of biocatalysts can be divided into two types: the effect which arises due to contact between the dissolved solvent and the biocatalyst on a molecular level (molecular toxicity), and the effect which arises due to direct contact between the biocatalyst and the aqueous/organic interface (interfacial toxicity). A rule of thumb has emerged for determining solvent biocompatibility which is based on the value of the LogP of the solvent, defined as the logarithm of the partition coefficient of the solvent in a water/1-octanol two-phase system. Broadly, a solvent is expected to be biocompatible if the LogP < 2 and incompatible if LogP > 4 (Laane et al., 1987) and for values between 2 and 4 the solvent effects are considered unpredictable. This rule tends to be more successful in predicting biocompatibility of miscible cosolvents. However, it may be still effective in determining the biocompatibility of an immiscible solvent since the LogP is inversely correlated with the saturation concentration of the solvents in water. Interfacial toxicity of solvents is dependent on other solvent properties such as polarity and interfacial tension (Ross et al., 2000). Despite the amount of research carried out in this area many exceptions

seem to exist to the general rules which have been proposed, and effects of solvents on biocatalysts have to be studied on a case by case basis. Although the direct relation between the amount of enzyme inactivated and total interfacial area in a biphasic system has been established (Ghatorae et al., 1994), a rigorous investigation of the main factors that influence the overall rate of interfacial inactivation of enzymes in emulsion reactors as a function of operating parameters is lacking. This is likely due to the difficulty in characterisation of emulsion systems, particularly in obtaining accurate measurements of droplet size distributions and droplet lifetime.

To reduce interfacial inactivation effects, membrane bioreactors can be used whereby the aqueous and organic liquid phases are kept separate by a hydrophilic or hydrophobic membrane. The advantage of such systems is that the biocatalyst in the aqueous phase does not directly contact the organic phase (Choi et al., 2000). However, membrane bioreactors tend to be complex to operate compared to emulsions since unwanted breakthrough of solvent through the membrane can occur and recycle rates through the two sides have to be carefully controlled. They are also not suited in cases where high interfacial areas are required for good mass transfer and are inflexible since bioreactors are usually designed for and dedicated to one reaction.

In multiphase systems, increasing the organic to aqueous phase ratio is an obvious way of increasing the volumetric productivity of a reaction that involves hydrophobic compounds since the concentration of substrate increases with respect to the total reaction volume. Limitations to the increase in phase ratio arise from practical difficulties in achieving sufficient mixing of the two phases. Furthermore, modifying phase ratios can change the concentration of product that accumulates in the aqueous phase, for example when the product is insoluble in the organic phase. The variation in interfacial area with phase ratio affects the overall rate of mass transfer of substrate to the aqueous phase, and thus also the substrate aqueous phase concentration. Because of this, the optimum enzyme concentration applied in the aqueous phase will almost certainly change with phase ratio as well as the overall rate of enzyme inactivation by the interfacial mechanism (Ghatorae et al., 1994). These types of interactions can be usefully studied by mathematical modelling of the biotransformation systems (*Chapter 2*). For liquid/liquid biphasic biotransformation systems, determination of model parameters and verification of model simulations can be carried using reactors such as the Lewis/stirred cell where interfacial areas are well defined (Woodley, 1991).

Preparative scale production of optically pure epoxides.

In recent years, large-scale preparation of optically pure epoxides has become an active field of research. Examples of the most successful preparative scale epoxide hydrolase mediated kinetic resolutions described

Table 1.2. Selected examples of preparative scale production of enantiopure epoxides by biocatalytic kinetic resolution

Biocatalyst	Epoxide Substrate	Epoxide conc.	[e.e. (%)] Yield (%)	Avg. Productivity † (Biocatalyst conc.)	Solvent/operating condition	Biocatalyst stability/inhibition
A. niger epoxide hydrolase (EH) [1] (whole cells)	styrene oxide	0.08 M 10 g L^{-1}	[96] 23 (S)	0.32 g L^{-1} h^{-1} (32 g cdw L^{-1})	27 ^{o}C, aqueous phase + 2% v/v ethanol	product inhibition for substrate conc.> 0.02 M
A. niger EH [2] (partially purified enzyme)	*p*-nitrostyrene oxide	0.33 M 54 g L^{-1}	[99] 49 (S)	3.7 g L^{-1} h^{-1} (51 g L^{-1} at 0.02 U mg^{-1})	25 ^{o}C, aqueous phase + 20 % DMSO (solid substrate as 2nd phase)	enzyme inactivation for substrate conc.> 0.35 M
A. niger EH [3] (partially purified enzyme)	2-pyridyl oxirane	0.082 M 10 g L^{-1}	[99] 27* (S)	0.33 g L^{-1} h^{-1} (0.5 g L^{-1} at 23 U mg^{-1})	4 ^{o}C, single aqueous phase	low temperature required for biocatalyst stability
A. radiobacter wild-type EH [4] (cell free extract)	2-pyridyl oxirane	0.127 M 15.4 g L^{-1}	[>99] 34.5* (S)	2 g L^{-1} h^{-1} (0.07 g L^{-1} at 13 U mg^{-1})	28 ^{o}C, single aqueous phase	product inhibition for substrate conc.> 0.2 M
A. radiobacter Y215F mutant EH [5] (cell free extract)	2-pyridyl oxirane	0.127 M 15.4 g L^{-1}	[98] 36* (S)	0.8 g L^{-1} h^{-1} (0.98 g L^{-1} at 0.11 U mg^{-1})	28 ^{o}C, single aqueous phase	product inhibition K_i (racemic) = 0.12 M
A. niger LCP521 EH [6] (cell free extract)	*p*-bromo-α-methyl styrene oxide	0.38 M 80 g L^{-1}	[99.7] 39* (S)	0.16 g L^{-1} h^{-1} 4.6 g L^{-1}	4 ^{o}C, biphasic system (liquid substrate as 2nd phase)	low temperature required for biocatalyst stability
Rhodoturula glutinis ATCC 201718 EH (whole cells) [7]	1,2-epoxy hexane	1.1 M	[98] 24 (S)	2.5 g L^{-1} h^{-1} (22 g cdw L^{-1})	25 ^{o}C, biphasic membrane bioreactor (substrate in dodecane)	product inhibition K_i = 0.05 M (continuous diol removal by extraction across membrane)

Table 1.2. continued.

Biocatalyst	Epoxide substrate	Epoxide conc.	[e.e. (%)] Yield (%)	Avg Productivity † (Biocatalyst conc.)	Solvent/operating condition	Biocatalyst stability/inhibition
A. niger EH [8] (cell free extract)	4-isobutyl-α-methyl styrene oxide	0.263 M 50g L^{-1}	[96] (S)	0.04 g L^{-1} h^{-1} (87 g L^{-1})	4 °C, biphasic system (substrate as 2nd liquid phase)	low temperature required for biocatalyst stability
S. tuberosum EH [9] (cell free extract)	*p*-chloro styrene oxide	0.2 M 30.6 g L^{-1}	[99] 47* (R)	0.84 g L^{-1} h^{-1} (33.6 g L^{-1} at 0.037 U mg^{-1})	0 °C, pH 6.7, biphasic system (substrate as 2nd phase)	inhibition at substrate conc.> 0.2 M
A. niger EH [10] (cell free extract)	*p*-chloro styrene oxide	2 M 306 g L^{-1}	[99] 47* (S)	26g L^{-1} h^{-1} (255 g L^{-1} at 0.23 U mg^{-1})	0 °C, pH 7 biphasic system (substrate as 2nd phase)	product inhibition at substrate conc. > 0.4 M; low temp. required for reasonable biocatalyst stability
A. radiobacter EH [11] (partially purified enzyme)	styrene oxide	0.324 M (39 g L^{-1})	[96] 30 (S)	0.27 g $L_{(tot)}^{-1}$ h^{-1} (0.014 g L^{-1} at 7.5 U mg^{-1})	30 °C, biphasic system 30 % v/v octane/buffer	product inhibition K_i *(S)*-diol = 0.03 M. Enzyme stability reduced under operating conditions.
Bacillus megaterium EH [12] (whole cells)	Phenyl glycidyl ether	0.06 M (9 g L^{-1})	[99.5] 25.6 (S)	0.14 g L^{-1} h^{-1} (30 g cdw L^{-1} at 0.003 U mg^{-1})	30 °C, aqueous phase + 5 % v/v DMSO	product inhibition at substrate conc.>0.06 M

† calculated on the basis of total reactor volume; *isolated yield

1 Pedragosa-Moreau et al.,1993; **2** Morrisseau et al., 1997; **3** Genzel et al., 2001b; **4**, **5** Genzel et al., 2001a, substrate solubility = 0.45 M (55 g L^{-1}); **6** Cleij et al., 1998; **7** Choi et al., 2000; **8** Cleij et al., 1999; **9, 10** Manoj et al., 2001; **11** Baldascini et al., 2001; **12** Tang et al., 2001

Table 1.3. Selected examples of preparative scale production of enantiopure epoxides by biocatalytic epoxidation and chemo-catalyzed hydrolytic kinetic resolution

Bio/catalyst	Epoxide	Epoxide conc.	[e.e. (%)]. Yield (%)	Avg. Productivity† (Biocatalyst conc.)	Solvent/operating condition	Stability
E.coli JM101(pSPZ10) [13]	styrene oxide	0.184M	[99.5]	2.2 g L^{-1} h^{-1}	organic/aqueous biphasic system 50 % v/v bis (2-ethylhexyl) phthalate	monooxygenase inhibition by styrene oxide. Cell growth inhibition by 2-phenylethanol (by-product). Toxicity to cells of styrene at organic phase conc. above 2 % v/v.
E.coli JM101(pSPZ10) [14]	styrene oxide	0.27 M	[>99] 49.7 (S)	1 g L^{-1} h^{-1}	organic/aqueous biphasic system: 50 % v/v bis (2-ethylhexyl) phthalate	
(salen)Co(III)(OAc) [15]	styrene oxide	(22.8 ml styrene oxide + 2 ml THF +2 ml water)	[>99] 44 * (R)	0.15 g h^{-1}	8 % v/v THF, temp. increased from 0 ^{o}C to room temp. during reaction	no product inhibition, catalyst recyclable

† calculated on the basis of total reactor volume; *isolated yield

13 Panke et al., 2000 (2L scale) ; **14** Panke et al., 2002 (30 L scale) ; **15** Schaus et al., 2002

in the literature to date is presented in Table 1.2. The most successful biocatalytic epoxidation and chemical preparative scale hydrolytic reactions are given for comparison in Table 1.3. The tables report the average reaction productivity, substrate concentrations, enantioselectivity, yield, and reaction type (single or multiphase) and give indications of biocatalyst stability and inhibition effects noted for the reaction systems. In all examples of kinetic resolution a high enantiomeric excess of the desired epoxide enantiomer could be obtained by letting the reaction proceed to sufficient conversion. For these resolution reactions the yield is limited to 50% by definition and yields approaching this maximum value were achieved in some examples, though often using very high biocatalyst concentrations. In nearly all cases the hydrolysis of the (*R*)-enantiomer occurred preferentially, yielding the enantiopure (*S*)-enantiomer. Organic solvents have been employed when the substrates are poorly soluble in water and in this manner high epoxide concentrations have been applied (entries [1], [2], [7], [11], [12]). In some cases ([2], [6], [8], [9], [10]), the liquid substrate constitutes a separate organic phase. Pyridyloxiranes are unusual epoxides which have the advantage of being reasonably soluble in aqueous solution (0.5 M) and these have been used in single phase systems to concentrations of 0.13 M without addition of cosolvents (entries [3], [4], [5]). Such single phase systems, however, offer no advantages for downstream recovery of the biocatalyst or epoxide product. In nearly all cases product inhibition has been identified as a limitation to using high epoxide concentrations, and this effect is generally attributed to inhibition by the formed diol. The application of a membrane bioreactor to enable use of high substrate concentrations in an apolar solvent, avoiding inactivation of the biocatalyst by direct contact with the organic phase, and allowing removal of the inhibitory diol, showed high productivity although overall yield was low (entry [7]). In these preparative scale examples average productivities range from 0.16 to 26 g L^{-1} h^{-1}, although direct comparison between the different systems is difficult since the biocatalyst concentrations used in the different examples vary greatly. In entry [11] a further increase in productivity would easily be possible by increasing the organic phase fraction containing substrate. Overall it seems that biocatalyst productivity remains low and appears not to have been optimised. Clearly, maintaining high biocatalyst activity during the biotransformation is a key issue in each of the examples listed. Biocatalyst inhibition is also a recurrent problem and in some cases the reactions are carried out at 4 ^{o}C to increase biocatalyst stability ([3], [4], [6], [8], [9], [10]), something which would likely be a disadvantage at an industrial scale. Before successful industrial application of these systems can occur, the stability and reusability of the biocatalyst needs to be determined.

Recently, the production of enantiopure styrene oxide has been carried out at the pilot-scale (30 L) (Panke et al., 2002) using recombinant *E.coli* expressing a styrene monooxygenase. The biotransformation was carried out

in a two-phase system for the common reasons outlined above. The concentration of the substrate, styrene was limited to 2% v/v in the organic phase since it is not tolerated by *E. coli* cells. The organic solvent was chosen to keep the aqueous concentration of the formed styrene oxide and 2-phenylethanol (a by product) in the aqueous phase low, since they both have an inhibiting effect. A specific inactivating effect of epoxides on alkene monooxygenases has been reported in many other cases (Habets-Crutzen and de Bont, 1985; Prichanont et al., 1998; Stanley et al., 1992) suggesting that due to their extreme reactivity, epoxides can affect biocatalyst stability by enzyme covalent modification by a common mechanism. Despite these inhibition effects, reasonably high concentrations of the formed epoxide accumulate in the organic phase. Volumetric productivity is also good at optimal cell densities, but scale-up of the reaction to 100 L already shows that factors such as the rate of substrate transfer from the organic phase and oxygen supply to the cells can become limiting (Table 1.3 entries 13 and 14).

Schaus et al. (2002) have recently reported on the practical hydrolytic kinetic resolution (HKR) of terminal epoxides using a chiral (salen) Co^{III}(OAc) complex. In this application the epoxide concentration used was generally very high since water was added only as a reactant. Small amounts of organic solvents such as Tetrahydrofuran (THF) were added for poorly aqueous soluble epoxides. The catalyst complex could be recycled without appreciable loss of activity and no product inhibition was observed. From a practical point of view, a disadvantage of this method is that the reaction temperature is required to be initially 0 ^{o}C and reaches room temperature during the conversion. The HKR of propylene oxide by the same catalyst has apparently been carried out at the multi-hundred kilogram scale at ChiRex, yielding enantiopure epoxide and 1,2-diol (Jacobsen, 2000). Ultimately, the choice between chemical and biocatalytic resolution methods of epoxides will be dictated by the required enantioselectivity for a specific epoxide intermediate and overall process economics.

Enzyme structure and stability

Enzyme stability is of primary importance in bioprocesses in order to maximise biocatalyst productivity so as to operate biotransformations at low cost. Correct enzyme function requires a correctly folded enzyme molecule. Protein folding is determined by a balance between conformational entropy which opposes the folded state and enthalpic and other entropic factors which favour the folded state (Mozhaev, 1993). Enzymes fold to maximise favourable interactions forming tightly packed hydrophobic cores exposing hydrophilic groups on the surface. Hydrophobic interactions greatly contribute to formation of the compact folded state due to the increase in

entropy of the water molecules which are released from the hydrophobic side groups of the polypeptide chain upon folding. Hydrogen bonds also play an important role in the stabilisation of enzyme secondary structure and together with electrostatic and dispersive forces all these interactions act to determine the structure of enzymes in a given environment. Since the stabilising and destabilising interactions compensate one another to a large extent, the native structure of a protein is only marginally stable. The Gibbs free energy for the transition from native to denatured state is only about –20 kJ mol^{-1} (Mozhaev, 1993) implying that even small changes in the environment of the enzyme can cause structural unfolding and loss of activity.

Enzyme inactivation

Causes of loss of enzyme activity include enzyme denaturation at high temperatures and extremes of pH, covalent enzyme modification by chemical species, enzyme adsorption to interfaces and inactivation due to shear which arises from structural damage from fluid forces in agitated systems. Studies of enzyme stability, particularly thermostability, are frequently conducted in the absence of substrate and product, under non-reacting conditions. However, it is known that enzymes are frequently stabilised in the presence of substrates, and similarly substrates can also interact with the enzyme to cause inactivation (Illanes et al., 1996). The increased application of enzymes in non-conventional media, for example in the presence of organic solvents, means that studying enzyme stability under representative process conditions is becoming increasingly important.

Enzyme stability is characterised by both thermodynamic and kinetic stability. Thermodynamic stability describes the reversible unfolding of enzymes molecules from the native state (N) to the unfolded state (U) under denaturing conditions (Mozhaev, 1993):

$$N \rightleftharpoons U \tag{1.4}$$

Differential scanning microcalorimetry (DSC) and circular dichroism (described below) can be used to determine the enthalpy of unfolding and the enzyme melting temperature (T_m) for such reversible unfolding. Assessment of the thermodynamic stability of enzymes using these techniques is particularly useful to compare the stability of different enzymes, or mutants of the same enzyme. However, thermodynamic stability can not easily be translated to kinetic enzyme stability, which concerns the time dependent irreversible enzyme inactivation under a set of conditions (Schein, 1990).

Irreversible inactivation involves a transition to an inactive form of the enzyme (I). In the simplest description, irreversible inactivation occurs by a first order irreversible unimolecular reaction,

$$N \xrightarrow{k_{obs}} I \tag{1.5}$$

and the rate of change of enzyme activity is described by the first order rate equation,

$$\frac{dN}{dt} = -k_{obs}N\,. \tag{1.6}$$

Frequently, irreversible inactivation is assumed to proceed by a two step mechanism comprising reversible unfolding to an intermediate denatured state (D), followed by an irreversible transition to the inactive form (Mozhaev, 1993),

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} D \xrightarrow{k_i} I \tag{1.7}$$

During thermoinactivation, for example, the last step is usually aggregation of the unfolded enzyme form, and under other denaturing conditions may involve covalent modification of labile amino acid residues, loss of cofactor or incorrect refolding of the denatured state. The rate constant for overall inactivation is (Sterner and Liebl, 2001),

$$k_{obs} = \frac{(k_u k_i)}{(k_f + k_i)} \tag{1.8}$$

Operational stability of enzymes is frequently quoted in terms of half-life ($t_{1/2}$) which is calculated by, $t_{½} = -\frac{\ln(0.5)}{k_{obs}}$. Determining the kinetics of enzyme inactivation is important to be able to take into account enzyme inactivation in bioprocess modelling.

Elucidation of the physical mechanism of inactivation is fundamental in order to devise targeted strategies to reduce inactivation. Enzyme inactivation can proceed by dissociation into subunits (quaternary structure), chemical modification (primary structure) and denaturation/conformational change (secondary and tertiary structure) which may then lead to enzyme aggregation and precipitation from solution. It is difficult to relate changes in activity to changes occurring in enzyme structure especially since the activity measured at any point in time can be the sum of contributions of any partially active enzyme forms (Polakovic, 1998). However, in systems where kinetics of substrate turnover are well-described, it is possible to differentiate between a change in substrate binding and a change in catalytic turnover rate and this can allow to distinguish between different causes of inactivation. In addition, following changes in enzyme structure by biophysical methods while measuring activity in parallel can be particularly informative about the inactivation mechanism.

Biophysical techniques for studying enzyme degradation

As described above, the loss of enzyme activity is always accompanied by a change in enzyme structure. This can be either a large conformational change, or a localised structural or chemical modification. Various biophysical techniques have been developed for monitoring these changes enabling fundamental studies of inactivation mechanisms. These techniques include UV absorption, circular dichroism (CD), fluorescence spectroscopy, mass spectrometry, peptide mapping and microcalorimetry (Schmid, 1997). Each technique is particularly suited for detecting specific enzyme structural modifications (Table 1.4) and can be used to determine enzyme thermodynamic stability by monitoring reversible unfolding under denaturing conditions. A brief description of the techniques used for work described in this thesis follows here.

CD measures the difference in adsorption between the two rotations of circularly polarised light by asymmetric molecules such as proteins, and so the CD spectrum is dependent on the conformation of the asymmetric molecule. The technique is non destructive and requires only small amounts of material. CD in the far-UV region (190-240 nm) is dominated by adsorption by the peptide group linking the amino acid residues, so that features of secondary structure such as α helices and β sheets give rise to characteristic spectra (Woody, 1995). The technique is frequently used to predict the secondary structure of proteins of unknown structure by comparison with reference spectra, and it is also a powerful technique for analysing changes in protein secondary structure. For example, far-UV CD can be used to follow thermal denaturation of proteins by following the change in CD signal at a fixed wavelength with temperature. We were particularly interested in using this technique to monitor large conformational changes in secondary structure for enzymes incubated in liquid/liquid biphasic systems (*Chapter 3*). Far-UV CD has also been a useful technique for studying the conformation of proteins adsorbed onto solid phases (Norde and Zoungrana, 1998). This has been possible since the solid phases can be made optically neutral. On the other hand, analysis of the structure of proteins adsorbed at liquid-liquid interfaces has been hindered by the physical instability of such surfaces and optical reflection at the interfaces. For this reason, in our experiments, we were limited to following changes in structure of soluble enzyme rather than enzyme adsorbed at the liquid/liquid interface. Recently external reflection circular dichroism has been presented as a novel technique to study the structure of proteins at the air/liquid interface (de Jongh and Meinders, 2002). This technique allows separation of the optical effects due to reflection and those due to molecular properties of the protein. The experimental set-up, however, is complex and sensitive to experimental imperfections so further improvements in the technique are necessary before unequivocal information on protein conformation can be obtained. In the near-UV range (250-300nm)

Table 1.4. Selected biophysical techniques used for monitoring enzyme degradation (chemical and structural changes)

Biophysical Technique	Structural features monitored	Information derived	Advantages *Disadvantages*	Ref
UV Absorption	- Tertiary structure - Ligand binding	- Protein conc. - Conformational changes	- Non-destructive - *Spectra can be difficult to interpret* - *Interference by chromogenic species*	Schmid, 1997
CD Far-UV	- Secondary structure	- Large conformational changes - Thermostability (T_m)	- Non-destructive - Low enzyme conc. (~ 0.1mg ml^{-1}) - *Low sensitivity to localised structural changes* - *Interference by chromogenic species*	Woody, 1995
CD Near-UV	- Tertiary structure - Ligand binding	- Localised conformational changes - Region of ligand binding - Ligand binding constants	- Non-destructive - Localised changes identified - *High enzyme conc. required (~2mg ml^{-1})* - *Interference by chromogenic species*	Woody and Dunker, 1996
Steady State Fluorescence	- Tertiary structure - Ligand binding	- Conformational changes - Unfolding	- Non-destructive - Low protein conc. sufficient (0.01mg ml^{-1}) - *Interference by fluorescing species*	Schmid, 1997
Peptide mapping	- Primary structure	- Covalent modifications - Region of modification	- Precise knowledge of the place of modification - *Labour intensive*	Stone et al., 1990
ESI-MS	- Ligand binding	- Molecular mass	- Accurate change in mass allows identification of ligand	Jardine, 1990
Microcalorimetry	- Unfolding	- Enthalpy of unfolding - Thermostability (T_m)	- Determines relative protein stability in different environments	Wadso, 1997

the CD spectrum is dominated by contributions made by the aromatic side chains of Phe, Tyr and Trp residues and is sensitive to the spatial arrangement of these side chains, so spectra reflect the tertiary structure of proteins (Woody and Dunker, 1996). Small conformational changes and ligand binding can be studied by following spectral changes in this region.

The fluorescence of proteins originates from Phe, Tyr and Trp residues but is often dominated by Trp when present (Schmid, 1997). Fluorescence spectra are sensitive to changes in the environment of these fluorophores making it a useful technique to monitor global protein unfolding. By comparing fluorescence spectra of single tryptophan mutants more site specific structural information can also be derived. Changes in emission intensity and shifts in the wavelength of maximal emission closer to 350 nm, which is the maximum for free tryptophan in solution, are observed upon unfolding. Measuring changes in fluorescence is commonly used to follow protein denaturation at high temperatures and in the presence of denaturants

Mass spectrometry by electrospray ionization (ESI) is a technique which allows the determination of protein molecular weights to high accuracy. Typically, relative masses in the range of 5 to 40 kDa can be obtained with an accuracy of 0.01% (Edmonds and Smith, 1990). The technique is particularly suited for identifying covalent and non-covalent protein-ligand complexes which may be formed upon inactivation (Pramanik et al., 1998). To be useful in identifying such complexes the technique must be optimised, especially regarding the sample preparation which can involve the use of cosolvents and acidic conditions, which may cause the complexes to be destroyed.

Improving enzyme stability

The simplest way of improving enzyme operational stability is by medium engineering. This involves optimising the operating temperature, choice of buffer and its concentration and working pH to achieve maximum enzyme stability. Various protective agents can also be added to increase stability. Commonly, glycerol is used to stabilise enzymes during storage, and 2-mercaptoethanol can be used to prevent intermolecular sulfydryl cross-linking, while EDTA can be used to chelate metal ions which can cause protein oxidation (Schein, 1990).

Many different strategies can be employed to further improve enzyme stability. The most common strategy used in industrial applications is immobilisation of the enzyme onto a solid carrier. Immobilisation usually functions by resisting protein unfolding and many examples in which enzyme operational stability has been successfully increased have been reported. Apart from increased stability, biocatalyst immobilisation onto a solid carrier offers the added advantage of facilitating the recovery of the biocatalyst from the reaction liquor so that it can be recycled. Immobilisation can be carried out by physical adsorption, gel entrapment, or

covalent attachment onto a carrier. The choice of immobilisation method will depend on the bioprocess conditions, taking into account the partitioning of substrate and product within the immobilisation matrix, and the perceived cause of inactivation. For example, in multiphase liquid systems, immobilisation of biocatalysts inside hydrophilic porous supports can help to reduce direct contact between the biocatalyst and the organic apolar liquid at the phase interface, thereby reducing interfacial inactivation. Disadvantages of immobilisation include the occurrence of diffusion limitations within the carrier porous particles. Mass transfer limitations result in an inefficient use of the biocatalyst and may cause a significant reduction in reaction selectivity (Barros et al., 2000), which is particularly relevant for kinetic resolution reactions. To reduce these negative effects the biocatalyst catalyst loading on the carrier particles has to be carefully optimised. An alternative approach could be to immobilise the biocatalyst on the outside of the carrier particles, but in this case the protection against interfacial inactivation would be less effective and the specific activity of the biocatalyst-carrier complex would be greatly reduced. Bioprocess modelling is invaluable in the study of the interaction between mass transfer and reaction in immobilised systems. Mateo et al (2003) described the covalent immobilisation of *Aspergillus niger* epoxide hydrolase onto an epoxy activated support. The immobilised biocatalyst could be reused for 12 successive batch epoxide resolution reactions with only a small loss of activity. The loading of enzyme onto the carrier was limited to 40 mg g^{-1} of (wet) support to avoid diffusional limitations and conversion kinetics were found to be changed with respect to the soluble enzyme, although this resulted in a favourable increase in enantioseletcivity of the immobilised enzyme.

Recently, the use of cross linked enzyme crystals (CLECs) has emerged as a very promising method for increasing enzyme stability (Margolin, 1996). CLECs are prepared by crystallisation of the enzyme followed by cross-linking with a bifunctional reagent, usually glutaraldehyde, which results in an insoluble and porous solid catalyst. This technique exploits the stabilising effects of both inter- and intramolecular cross-links which are formed between the amine groups of lysines. Compared to the soluble enzyme forms, CLECs have been shown to possess higher thermostability, proteolytic stability and increased resistance to organic solvents (Govardhan, 1999). Additionally CLECs can easily be recovered from reaction media by filtration for reuse. Disadvantages in the use of CLECs are that the specific activity is often greatly reduced compared to the soluble enzyme. Compared to enzymes immobilised on solid supports, however, specific activity is often higher and the expensive carrier material is not required. Similarly to immobilised enzymes, mass transfer limitations may occur within the porous crystal matrix and this can have important consequences on the effective enantioselectivity of the biocatalyst (Tischer

and Kasche, 1999). Use of CLECs at an industrial scale (100 L) in a two-phase system for the kinetic resolution of sec-phenethyl acetate has been demonstrated (Collins et al., 1998). Reusability of the CLECs in successive batch reactions indicated that most of the activity that was lost between successive batches was due to loss of catalyst mass at the filtration stage in the recovery step, indicating that the size of the CLECs is an important physical characteristic for their use at the industrial scale.

Crystallisation of an enzyme with retention of activity is not always possible. An alternative method which also exploits cross-linking for enzyme stabilisation is the creation of cross-linked enzyme aggregates (CLEAs) (Cao et al, 2001). This method does away with the necessity of crystallisation since cross-linking is carried out on enzymes which are aggregated under non-denaturing conditions. CLEAs of penicillin acylase have been shown to be more stable in the presence of organic cosolvents than the conventionally immobilised enzyme, but the general applicability of this method remains to be demonstrated.

The preferred strategy for maintaining biocatalyst activity under reaction conditions would be to increase the intrinsic stability of the biocatalyst (enzyme). The use of enzymes from thermophilic organisms, which show high thermostability, has been advocated since higher thermostability has been correlated with increased resistance to other mechanisms of denaturation, such as cosolvent effects (Owusu and Cowan, 1989). However, there is often a trade-off between thermostability and activity, since enzymes from thermophilic organisms generally have low activities at room temperatures (Carrea and Colombo, 2000).

Site-directed mutagenesis (SDM) and more recently directed evolution are emerging as powerful techniques to improve enzyme characteristics (Bornscheuer and Pohl, 2001). The former technique requires in-depth knowledge of enzyme structure-function relationships in order to be able to predict mutations will have the desired effect on enzyme function (van den Burg and Eijsink, 2002). The mutation of one active site tyrosine residue of the epoxide hydrolase used for work in this thesis resulted in mutants (Y215F and Y152F) with much higher enantioselectivity for the conversion of some substrates (Rink et al., 1999), which is clearly highly desirable for industrial application. Interestingly, these mutations also increased enzyme stability against extremes of pH (Rink et al., 2000) which prompted us to also investigate the thermostability of the different enzyme forms (*Chapter 5*).

In contrast to SDM, directed evolution does not require much detailed knowledge of the enzyme structure-function relationships since mutations are introduced randomly. However, it does require a robust screening assay which accurately reflects the properties of interest (Arnold, 2001; Petrounia and Arnold, 2000). Directed evolution has successfully been used to improve enzyme substrate range and to increase, or alternatively invert,

enzyme enantioselectivity (Zha et al., 2001). The development of quantitative high throughput methods to screen for catalyst enantioselectivity remains a challenge, and recent examples of such methods are reviewed by Reetz (2002). Improvement of enzyme thermostability by directed evolution has also been demonstrated (Gonzales-Blasco et al., 2000) and again, screening for increased enzyme thermostability should be accompanied by screening for reasonable activity at low temperatures. Using the technique of DNA shuffling, Ness et al. (2001) successfully improved subtilisin activity at room temperature, thermostability and stability against organic cosolvents in a single enzyme clone. Analysis of the mutations in evolved enzymes show that these are often not situated in the active site and that mutations farther away seem to have long ranging structural effects that can affect enzyme performance (Petrounia and Arnold, 2000). For this reason, rational design of improved stability is particularly challenging since many different interactions contribute to the stability of proteins.

Although there is a certain correlation between increased enzyme thermostability and increased operational stability even in the presence of organic solvents, it is important to appreciate that resistance to inactivation due to different mechanisms will not necessarily be directly correlated. For example, a percentage increase in thermostability may not give a discrete improvement in the resistance to inactivation at a liquid/liquid interface. Nevertheless, it is expected that these two techniques will play a major role in improving enzyme properties especially in regards to their use as industrial catalysts, and in the future this will be focussed to a large extent on the increase of their operational stability.

The techniques which are used to stabilise enzymes should be evaluated in terms of their effects on the whole biocatalytic process. For example, while immobilisation may increase stability, the introduction of a large proportion of solid phase may cause unwanted problems in downstream separation. Moreover, the optimum operating pH, and temperature need to be redefined for an immobilised enzyme preparation. Even when mutants are created which have improved characteristics such as higher enantioselectivity – their use in the whole bioprocess must be re-evaluated. This is because while the desired characteristic of the biocatalyst might be improved, unexpected effects on other properties may emerge. The creation of the site directed mutants of the epoxide hydrolase used for the work described in this thesis is a good example of this. The active site mutant Y215F showed a much higher enantioselectivity than the wild-type enzyme. Although the enantioselectivity was higher, the K_m values for the conversion of epoxide enantiomers were increased 100 to 1000-fold compared to the wild-type enzyme (Rink et al., 1999). The consequences of this for bioprocess design could be two-fold. Firstly, in a biphasic application maintaining high enough substrate concentration in the aqueous phase in order to maintain high overall conversion rates could become

problematic, thus necessitating higher mass transfer rates (*Chapter 2*). Secondly, it was found that although the K_m values for substrate binding to the enzyme active site were increased, product binding (inhibition) constants did not vary much, thereby increasing the relative importance of product inhibition at lower diol product concentrations (*Chapter 4*), which is clearly undesirable. Koeller and Wong (2001) suggest that in a strategy for the development of effective biocatalysts, process development comes as the final step after the biocatalyst has been optimised. It can be argued that evaluation of biocatalyst characteristics in relation to bioprocess optimisation should be integrated with mutant screening as early as possible in bioprocess development, so that the evolution of the biocatalyst is directed towards optimising overall bioprocess performance rather than specific characteristics of the biocatalyst alone. Rapid screening facilities to combine high throughput evaluation of catalyst variants with regard to overall bioprocess performance have already been demonstrated for whole cell biocatalysts (Lye et al., 2003).

Aim and Outline of this Thesis

Research on the epoxide hydrolase from *Agrobacterium radiobacter* AD1 carried out in our and other research groups has included a detailed study of enzyme function (van den Wijngaard et al., 1989), catalytic and kinetic mechanism (Rink and Janssen, 1999) and structure by X-ray crystallography (Nardini et al., 2000). Recognition of the potential of the use of this enzyme in organic synthesis for the production of optically pure epoxides initiated the study of enzyme substrate range and an exploration of biocatalytic applications of the enzyme (Lutje Spelberg et al., 1998, 2002a). The combination of all these studies allowed the rational design of mutant epoxide hydrolases with greatly increased enantioselectivity for the kinetic resolution of various industrially interesting epoxides (Rink et al., 1999). The work in this thesis is focussed on the application of the wild-type and mutant epoxide hydrolases at a preparative scale, addressing the constraints which are identified in obtaining optically pure epoxides with high volumetric productivity, high enantiomeric excess and high biocatalyst productivity. The focus on epoxide hydrolase stability in this thesis is motivated by the fact that stability is a major determinant of bioprocess productivity. A fundamental study of the factors which affect epoxide hydrolase operational stability is carried out in order to formulate appropriate solutions for maximising enzyme stability.

In *Chapter 2* the use of a two-liquid-phase system composed of octane and buffer is demonstrated in order to carry out the kinetic resolution with high epoxide concentrations and to reduce the loss of yield caused by spontaneous hydrolysis of the epoxide. Styrene oxide was used as model

substrate for this biphasic biotransformation. A process model describing the bioconversion in the biphasic system was developed and validated using a stirred cell reactor. The model was used to study the effect of mass transfer limitations on the apparent enantioselectivity of the kinetic resolutions.

Results from *Chapter 2* indicated that enzyme inactivation from dissolved octane in the aqueous phase was low, whereas inactivation due to contact between the enzyme and the organic/aqueous interface was greater. A study of enzyme interfacial inactivation occurring at the octane/buffer interface is the subject of *Chapter 3*. The influence of mixing intensity, enzyme concentration and presence of styrene oxide in the organic phase on the rate of interfacial inactivation are investigated. By measuring changes in the secondary structure of dissolved enzyme by CD, interfacial inactivation was proposed to involve the sequential steps of adsorption, enzyme structural rearrangement with loss of activity, desorption and aggregation and precipitation of desorbed enzyme molecules. Using a Lewis cell reactor, it was found that the rate of interfacial inactivation increased with increasing mixing intensity. This was attributed to an increase in the rate of eddy assisted desorption of inactivated enzyme from the interface which in turn allows enzyme in solution to adsorb and inactivate. The specific inactivation rate measured in an emulsion system was lower than in the stirred-cell even though interfacial area was higher. The presence of styrene oxide at concentrations above 250 mM in the octane liquid was shown to increase the rate of interfacial inactivation.

Chapter 4 describes a study of the effect of vicinal diols on epoxide hydrolase stability. Incubation of wild-type enzyme in aqueous solutions of diols caused irreversible enzyme inactivation. The inactivating effect was greater at higher diol concentrations. The inactivation occurred specifically with vicinal diols and was positively correlated with the inhibition effect of the vicinal diols, which are also competitive inhibitors of the enzyme. Active site specificity was demonstrated for the inactivation. Inactivation was not accompanied by large changes in enzyme structure but a smaller conformational change could be observed by near-UV CD spectroscopy. Interestingly, the Y215F active site mutant of this epoxide hydrolase was resistant to inactivation by diols.

The research described in *Chapter 5* was aimed at investigating the possibility of using cross-linked epoxide hydrolase crystals (CLEhCs) to carry out kinetic resolution reactions with high enzyme stability. Crystals of the epoxide hydrolase active site mutant (Y215F) were obtained by batch crystallisation using PEG as the precipitating agent. The enzyme crystals were cross-linked using glutaraldehyde. The CLEhCs were resistant to dissolution in buffer, were active and enantioselective and could be reused for successive epoxide hydrolysis reactions. The apparent enantioselectivity of the CLEhC preparation was lower than that of soluble enzyme for the

conversion of two epoxides tested. Nevertheless, epoxides with high e.e. and reasonable yield could be obtained using the CLEhCs.

Chapter 6 presents a summary of the results and an outlook for the different possibilities for production of optically pure epoxides by kinetic resolution of racemic mixtures.

Chapter 2

Effect of mass transfer limitations on the enzymatic kinetic resolution of epoxides in a two-liquid-phase system

Abstract

Optically active epoxides can be obtained by kinetic resolution of racemic mixtures using enantioselective epoxide hydrolases. To increase the productivity of the conversion of sparingly aqueous soluble epoxides, we have investigated the use of a two-phase aqueous/organic system. A kinetic model which takes into account interphase mass transfer, enzymatic reaction and enzyme inactivation was developed to describe epoxide conversion in the system by the epoxide hydrolase from *Agrobacterium radiobacter*. A Lewis cell was used to determine model parameters and results from resolutions carried out in the Lewis cell were compared to model predictions to validate the model. It was found that n-octane is a biocompatible immiscible solvent suitable for use as the organic phase. Good agreement between the model predictions and experimental data was found when the enzyme inactivation rate was fitted. Simulations showed that mass transfer limitations have to be avoided in order to maximize the yield of enantiomerically pure epoxide. Resolution of a 39 g L^{-1} solution of racemic styrene oxide in octane was successfully carried out in an emulsion batch reactor to obtain (*S*)-styrene oxide in high enantiomeric excess (>95% e.e.) with a yield of 30%.

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Introduction

Epoxides are important intermediates for the production of a wide range of fine-chemical and pharmaceutical products (Furuhashi, 1992). Biological activity of these products often lies with only one enantiomer and the potential toxic side effects of the other enantiomer have made their production in enantiomerically pure form increasingly important (Crosby, 1992).

Some successful chemocatalytic routes for the production of enantiomerically pure epoxides, such as the heavy metal complex catalyzed oxidation by Sharpless and Jacobsen/Katsuki, have been developed. However, these can be applied only for specific substrate structures (Besse and Veschambre, 1994; Tokunaga et al., 1997). Synthetic biocatalytic methods include alkene epoxidation by monooxygenases and reduction of α-haloketones to optically pure halohydrins (de Bont, 1993), and are usually cell-mediated processes since cofactors are required.

Enzymatic enantioselective degradation of racemic epoxides, which are relatively cheap to produce, has been recognized as an attractive alternative to these (bio)synthetic routes (Archer, 1997; Pedragosa-Moreau et al., 1996; Weijers and de Bont, 1999). Some epoxide hydrolases are capable of hydrolyzing epoxides enantioselectively to diols. According to a recent review on epoxide degrading enzymes (Swaving and de Bont, 1998), epoxide hydrolases are the most promising enzymes for biocatalytic production of enantiopure epoxides since no cofactors are involved; they have a broad substrate range; and both the optically pure epoxide and the diol can be used as chiral synthons.

We previously isolated an enantioselective epoxide hydrolase from *Agrobacterium radiobacter* AD1 (Rink et al., 1997), which hydrolyses a variety of terminal epoxides with a moderate to high enantioselectivity (Lutje Spelberg et al., 1998). Overexpression of epoxide hydrolase in *E. coli* has made this enzyme available in large quantities (Rink et al., 1997), increasing its potential for large-scale enantiopure epoxide preparation.

The productivity of this biocatalytic route is limited, however, by the low aqueous solubility of epoxides, which is typically a few grams per liter, the loss of epoxide due to chemical hydrolysis, and enzyme inhibition at high diol concentrations.

The use of cosolvents has been attempted to increase epoxide aqueous solubilities (Nellaiah et al., 1996). Alternatively, poorly soluble substrates can be applied as two-phase systems with the substrate as the second phase. For example, asymmetric hydrolysis by a fungal epoxide hydrolase, of a solid epoxide (50 g L^{-1}) as a suspension in buffer containing 20 % (v/v) dimethyl sulfoxide, has been demonstrated (Morisseau et al., 1997). However, enzyme inactivation seemed to be a limitation in this system. Substrates can also be applied in two-phase systems dissolved to high

concentrations in an apolar solvent. The solvent should not have deleterious effects on enzyme activity and should be chosen so that partitioning of substrate and product over the two phases facilitates downstream separation. If a solvent is toxic to the biocatalyst, a membrane can be employed to separate the biocatalyst from the organic solvent. A resolution of 1,2-epoxyhexane dissolved in dodecane at high concentrations using a recycle membrane reactor has been demonstrated with yeast cells, which allowed highly enantioselective hydrolysis of the epoxide (Choi et al., 1999).

In this chapter, we describe the use of a two-liquid-phase system for the resolution of racemic epoxides without using a membrane by selecting a suitable organic solvent. A two-phase system should be advantageous since the organic liquid can act as a reservoir of substrate, increasing bioreactor productivity. Furthermore, loss of epoxide by spontaneous, nonenantioselective chemical hydrolysis may be reduced by partitioning of a large proportion of the epoxide to the organic phase.

In heterogeneous systems of immobilized enzymes, mass transfer limitations through the carrier material can greatly reduce the effective enantioselectivity of a resolution (Barros et al., 2000; Matson et al., 1989). Mass transfer limitations also affect the effective enantiomeric ratio, E, observed for solid-to-solid kinetic resolutions (Straathof et al., 1998). In our system, styrene oxide was used as the model substrate for which the (*R*)-enantiomer is preferentially hydrolyzed with an E value of 16 (Lutje Spelberg et al., 1998). The kinetic mechanism for this substrate has been studied in detail (Rink and Janssen, 1998). Typical of enantioselective hydrolysis of epoxides by this epoxide hydrolase is that the enantiopure substrate (*S*) is rapidly degraded after the unwanted (*R*)-enantiomer is hydrolyzed. The same type of sequential conversion kinetics has been observed for the enantioselective hydrolysis of *tert*-butyloxirane and styrene oxide by microsomal epoxide hydrolase (Watabe et al, 1981; Wistuba and Schurig, 1992). Because of these complex kinetics it is expected that the relative rates of substrate interphase mass transfer, enzymatic reaction and enzyme inactivation are critical factors in determining the effective enantioselectivity of the conversion in the two-liquid-phase system. We therefore studied mass transfer and enzyme inactivation in a Lewis cell (Woodley et al., 1991), which has a well-defined interfacial area. We developed a mathematical model to describe the conversion of racemic epoxide in the system. The results of simulations showed that mass transfer limitations have to be avoided in order to obtain high yields of enantiomerically pure epoxide in short process times. Resolution of a concentrated solution of racemic styrene oxide in octane (39 g L^{-1}) was carried out to demonstrate the potential of using this two-phase system.

Modeling of two-phase enzymatic hydrolysis

The model describes interphase mass transfer, enzymatic reaction, spontaneous epoxide hydrolysis and enzyme inactivation. These processes are coupled through mass balances over both the aqueous and organic phases.

It is assumed that the enzymatic reaction takes place in the bulk of the aqueous phase and that mass transfer of the epoxide to the aqueous phase and hydrolysis occur sequentially. Assuming interfacial equilibrium, the (*S*) and (*R*)-enantiomer interfacial concentrations are given by:

$$S_i = S/m, \tag{2.1}$$

$$R_i = R/m, \tag{2.2}$$

where m is the partition coefficient.

Since m is high it is assumed that mass transfer resistances occur only in the aqueous phase. The flux of each enantiomer over the interface, J, can then be described based on an aqueous phase driving force using the aqueous phase mass transfer coefficient, k_a, which is assumed constant over the range of operating conditions:

$$J_S = k_a\left(S_i - S_a\right), \tag{2.3}$$

$$J_R = k_a\left(R_i - R_a\right). \tag{2.4}$$

As the (*R*) and (*S*)-enantiomers react in the bulk of the aqueous phase, transfer of (*R*) and (*S*) occurs from the organic phase to the aqueous phase. The diol is assumed to remain completely in the aqueous phase, therefore the change in diol aqueous concentration is described by:

$$\frac{dD_S}{dt} = -r_S, \tag{2.5}$$

$$\frac{dD_R}{dt} = -r_R. \tag{2.6}$$

The enzymatic conversion of both enantiomers of styrene oxide in the aqueous phase can be described by competitive Michaelis-Menten kinetics and competitive product inhibition, with a term also for spontaneous chemical hydrolysis (Lutje Spelberg et al., 1998):

$$r_S = -\frac{k_{cat}^S[E]S_a}{S_a + \left(\frac{R_a}{K_m^R} + \frac{D_S}{K_i^{D_S}} + \frac{D_R}{K_i^{D_R}} + 1\right)K_m^S} - k_c S_a, \tag{2.7}$$

$$r_R = -\frac{k_{cat}^R[E]R_a}{R_a + \left(\frac{S_a}{K_m^S} + \frac{D_S}{K_i^{D_S}} + \frac{D_R}{K_i^{D_R}} + 1\right)K_m^R} - k_c R_a. \tag{2.8}$$

Initial rate experiments were performed to determine competitive product inhibition constants for both enantiomers of the diol product. Kinetic constants are summarized in Table 2.1.

Enzyme inactivation is assumed to be described by a first-order process:

$$\frac{d[E]}{dt} = -k_d[E]. \tag{2.9}$$

A mass balance over the aqueous phase gives:

$$\frac{dS_a}{dt} = J_S\left(\frac{a}{1-\Phi}\right) + r_S, \tag{2.10}$$

$$\frac{dR_a}{dt} = J_R\left(\frac{a}{1-\Phi}\right) + r_R; \tag{2.11}$$

and for the organic phase:

$$\frac{dS}{dt} = -J_S\left(\frac{a}{\Phi}\right), \tag{2.12}$$

$$\frac{dR}{dt} = -J_R\left(\frac{a}{\Phi}\right). \tag{2.13}$$

Table 2.1. Kinetic constants for the conversion of styrene oxide by *A. radiobacter* AD1 epoxide hydrolase at 30 °C.

Enantiomer	K_m [a] (μM)	k_{cat} [a] (s^{-1})	k_c (s^{-1})	K_i (mM)
(*R*)-styrene oxide	0.6 ± 0.2	4.2 ± 0.3	9.54 x 10^{-6}	-
(*S*)-styrene oxide	25 ± 5	10.5 ± 1.0	9.54 x 10^{-6}	-
(*R*)-1-phenyl-1,2-ethane-diol	-	-	-	42 ± 10
(*S*)-1-phenyl-1,2-ethane-diol	-	-	-	29 ± 5

[a] Rink and Janssen, 1998. The value of k_c was determined at pH 7.5.

Materials and methods

Materials

Racemic styrene oxide (97%) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and n-octane (p.a. grade) from Fluka (Buchs, Switzerland). All other solvents used were at least reagent grade. (*R*)-*p*-nitrostyrene oxide (pNSO) was synthesized as described by Westkaemper and Hanzlik (1981). The aqueous phase was a buffer of composition 50 mM Tris-SO_4, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol.

Simulation and parameter estimation

Model simulations and parameter estimation was carried out using the software program Scientist™ (MicroMath Inc., Salt Lake City, UT).

Enzyme preparation

A partially purified enzyme was used throughout this work. Protein was expressed as described by Rink et al. (1997). Purification was carried out with a DE52 anion exchange column. The partially purified enzyme was dialyzed against TEMAG buffer (25 mM Tris-SO_4, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02 % sodium azide, and 10 % glycerol) and concentrated to 14 mg ml^{-1} (400 μM). The enzyme was stored at 4 ^{o}C.

Analytical Procedures

Epoxide concentrations in the octane phase were analyzed by diluting 100-200 μl samples in 2 ml octane, containing mesitylene (0.05 % v/v) as internal standard, and analyzed by GC. Epoxide in the water phase was analyzed after extracting 0.5 ml samples with 1 ml diethyl ether (containing mesitylene as internal standard) for 1 min. The top layer was dried on a short column of anhydrous Na_2SO_4 and then analyzed by GC.

Chiral analysis for Lewis cell experiments was performed with a HP 5890 GC equipped with a FID detector, using a Chiraldex G-TA capillary column, 50 m in length and 0.25 mm internal diameter. Injector and detector temperatures were 250 ^{o}C and isothermal operation was used at 120 ^{o}C. Chiral analysis for the emulsion experiment was performed with a HP6890 GC equipped with a FID detector, using a 25 m x 250 μm x 0.25 μm CP Chirasil-Dex CB column. Injector and detector temperatures were 250 ^{o}C. The oven was held at 100 ^{o}C for 5 mins after which the temperature was increased at 5 ^{o}C min^{-1} to 120 ^{o}C, at which it was held for 2 min. Nonchiral analysis was performed with the HP6890 GC, using a 0.2 mm x 25 m HP5 column. Injector and detector temperatures were 300 ^{o}C. The oven was held at 50 ^{o}C for 1 min after which the temperature was increased at 10 ^{o}C min^{-1} to 110 ^{o}C, at which it was held for 5 min.

Aqueous phase concentrations of 1-phenyl-1,2-ethanediol were measured by derivatisation with 2,2-dimethoxypropane to give a hemiacetal that was analysed by GC, as described elsewhere (Rink and Janssen, 1998).

Activity assays

Enzyme activity assays were performed in a Perkin Elmer Lambda Bio 40 UV/VIS spectrophotometer with a temperature-controlled cell holder by following the hydrolysis of the colorimetric substrate (*R*)-pNSO to its corresponding diol at 310 nm, for which extinction coefficients are ε_{310} = 4,289 $M^{-1}cm^{-1}$ and ε_{310} = 3,304 $M^{-1}cm^{-1}$, respectively. Typically, 1 ml of enzyme solution (approx. 0.06 μM) was placed in a 1 cm quartz cuvette and the reaction was started by adding up to 10 μl of a 10 mM (*R*)-pNSO

solution in acetonitrile. Conversion curves were numerically fitted to Michaelis-Menten kinetics to determine k_{cat} and K_m.

Solvent biocompatibility

To qualitatively compare the effect of the organic solvents on epoxide hydrolase activity, 2.5 ml of enzyme solution (approximately 4 μM) in 50 mM Tris buffer, pH 9, was incubated with 0.6 ml of organic liquid in closed test tubes and rotated end-over-end at a rate of 5 rpm at 30 ^{o}C. After 1 and 5 h, the phases were allowed to separate and the aqueous layer was assayed for enzyme activity by using pNSO as substrate. In this way, both inactivation by the interface and by dissolved organic solvent are included. To separately determine the effect of dissolved solvent on epoxide hydrolase activity, enzyme was incubated in buffer saturated with the solvents at 30 ^{o}C, but with no separate solvent phase. The remaining activity was tested after 1 and 5 h of incubation. A control incubation with no solvent was used to measure inactivation of the enzyme alone.

The Lewis Cell

The Lewis cell is a cylindrical vessel with two compartments separated by an interfacial plate which is attached to four baffles and contains a central hole to allow contact between the two phases (Fig. 2.1). Interfacial plates with holes of varying sizes were available. In all experiments the Lewis cell was thermostatted at 30 ^{o}C. Sample ports were present on the top and bottom of the cell to enable samples to be taken from either phase during operation. Each phase was mixed by an independently rotating Rushton impeller mounted in the middle of each compartment. Mixing time experiments (based on acid-base titrations in the presence of an indicator) showed that a stirring rate of 150 rpm was sufficient to obtain homogeneous phases.

Measurement of substrate interphase mass transfer rates and diol partitioning

The Lewis cell was filled with 97 ml of the aqueous buffer phase and n-octane was carefully placed on top so that the interface remained flat. Styrene oxide was added to the octane phase to 90 mM or 100 mM. Stirring in both phases was set to 150 rpm (counter-current). The epoxide aqueous phase concentration was followed in time by taking 0.5 ml samples through a septum in the bottom of the cell, which were analyzed as described above. After sampling, fresh buffer was added to replace any volume of the aqueous phase removed for analysis in order to maintain the interface positioned at the interfacial plate. The aqueous phase mass transfer coefficient (k_a) and the partition coefficient (m) were obtained by fitting the concentration profiles to mass transfer equations (2.1-2.4, 2.10-2.13), also taking into account spontaneous hydrolysis of the epoxide, using a least squares minimization

procedure of the software program Scientist^{TM}. Values are quoted at the 95% confidence level.

The effect of diol present in the aqueous phase on mass transfer and partitioning of epoxide was tested by repeating the same procedure in the Lewis cell as described above, with the aqueous phase initially at various concentrations of diol (10 mM, 22 mM and 50 mM).

Diol partitioning between the two phases was determined by equilibration experiments. In batch incubations, 3 ml of 10 mM or 100 mM aqueous solutions of diol were added to 3 ml of octane and mixed at 30 ^{o}C for 24 h. The phases were allowed to separate and the aqueous phase was tested for remaining diol concentration. The organic phase diol concentration after equilibration was calculated from a mass balance.

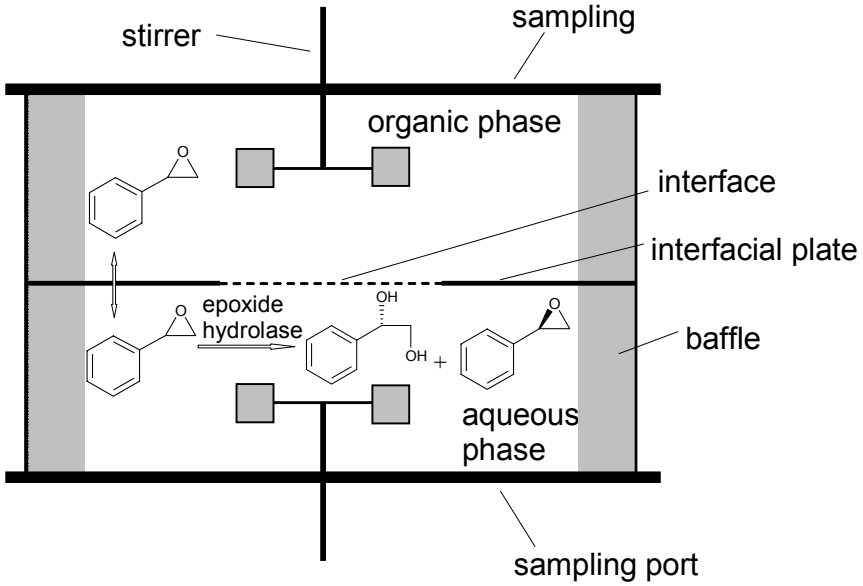

Figure 2.1. Diagram of the Lewis cell. Dimensions and operating conditions: height = 4.6 cm, diameter = 7.5 cm, upper and lower volumes 97 ml each, plate hole interfacial area = 22 cm^2, turbine diameter = 3.8 cm, blade height = 0.7 cm, blade width = 0.7 cm, baffle width = 0.7 cm, stirrer speed = 150 rpm.

Stability of epoxide hydrolase

The stability of epoxide hydrolase in buffer was tested by incubating a solution of epoxide hydrolase in buffer (0.11 μM) at 30 ^{o}C. Periodically, assays were performed with (*R*)-pNSO to determine the residual enzymatic activity.

To test for molecular toxicity of octane, epoxide hydrolase was incubated in a closed vessel in buffer (0.11 μM) which had previously been saturated with n-octane at 30 ^{o}C. Periodically, assays were performed with (*R*)-pNSO to determine the residual enzymatic activity.

The effect of interfacial contact with octane on enzyme activity was tested in the Lewis cell. The Lewis cell was filled as described above but

with no substrate present (0.11 μM enzyme concentration). The stirring speed was 150 rpm for both phases with stirrers rotating countercurrently. The interface was stabilized by an interfacial plate, which fixed the contact area at 22 cm^2. Periodically, assays were performed as follows: 0.5 ml of aqueous phase were withdrawn from the bottom of the Lewis cell and diluted by adding to 0.7 ml of buffer. The activity was tested by a pNSO assay as already described. An equal volume of buffer was replaced to the bottom phase directly after each sample was taken. The activities obtained were corrected for this dilution. First order inactivation rate constants were fitted to the inactivation curves with a least-squares minimizing procedure and are quoted at the 95% confidence level.

Kinetic resolution of styrene oxide in the Lewis Cell and the emulsion system

Resolution of a racemic mixture of styrene oxide in the Lewis cell was performed by filling the bottom of the cell with aqueous phase and injecting a known amount of enzyme, then placing the octane on top. Once the stirrers were in motion at 150 rpm, the reaction was initiated by injecting styrene oxide into the top layer to the desired concentration. Periodically, samples were taken of the octane phase and analyzed by chiral GC as described above. Aqueous phase concentrations were not monitored to limit the disturbance of the system.

The 200 ml vessel of the Lewis cell, without an interfacial plate and stirred by only one of the Rushton turbines positioned in the middle of the liquid, was used for the resolutions with an emulsion. The reactor was filled with 120 ml aqueous phase and epoxide hydrolase was added to the desired concentration. With the stirrer in motion, 30 ml ($\Phi = 0.2$) of octane made up to the desired concentration of styrene oxide were injected in the vessel to start the reaction. The stirring speed was set at 200 rpm. Periodically, samples were taken from the organic phase and analyzed by GC on a chiral column as described.

Results and Discussion

Choice of Solvent

Immiscible solvents can cause enzyme inactivation due to dissolved solvent molecules in the aqueous phase interacting with the enzyme (molecular toxicity) and due to direct contact of the enzyme with the interface (interfacial toxicity) (Bar, 1988). Seven solvents were compared for both interfacial and molecular toxicity to epoxide hydrolase by incubating enzyme solutions with each solvent present as a separate phase. The incubation test tubes were rotated slowly end-to-end. All solvents tested caused a decrease in enzymatic activity with time (Table 2.2), whereas the

control incubation, with no solvent, showed no change in activity over the tested time period. Octane had the least effect on enzyme activity, which decreased by only 20% after incubation for 5 h. It should be noted that octane and hexane appeared harder to emulsify than the other solvents and with these solvents only relatively small, flat interfaces were created during the gentle mixing, rather than droplets which were formed with all the other solvents. The inactivation caused by the dissolved part of each solvent was tested by incubating enzyme in buffer saturated with solvent, but with no separate solvent phase present. Dissolved solvent caused complete enzyme inactivation within 5 h for all solvents except hexane and octane. For octanol, the dissolved solvent inactivation effect was slightly greater than when a separate phase was also present. This may be explained if the dissolved octanol concentration did not reach the saturation concentration during the first hour of incubation under such gentle mixing conditions. For hexane and octane, the inactivation was greater when a free interface was present, indicating that molecular and interfacial toxicity effects could be cumulative. The log P of a solvent, the logarithm of the partition coefficient of the solvent in a standard mixture of 1-octanol and water, is a property often used for predicting biocompatibility (Laane et al., 1987), and is usually more indicative of dissolved solvent effects. The relative effects of the solvents correlated well with the biocompatibility criteria since octane and hexane, with log P > 4, had the least effect on enzyme activity, whereas solvents with a log P < 2 had the greatest inactivating effect. The low aqueous solubility of octane (Table 2.2) in water may account for its lower molecular toxicity. Octane was chosen as the best solvent because it had the least effect on enzyme activity. Furthermore, it is miscible with styrene oxide but is a poor solvent for the diol produced. The diol remains mostly in the aqueous phase (see below), with obvious advantages for downstream separation.

Table 2.2. Properties of organic solvents and their effect on epoxide hydrolase activity.

Solvent	Log P [a]	Aqueous solubility [a] at 25°C (M)	Molecular toxicity, % remaining activity after		Molecular and interfacial toxicity, % remaining activity after	
			1 h	5 h	1 h	5 h
i-Amyl acetate	-	-	<1	-	-	0
Diethylether	0.85	0.93	0	-	0	0
n-Butyl acetate	1.7	-	0	-	-	0
1-Hexanol	2.0	0.13	0	-	0	0
1-Octanol	2.8	4.5×10^{-3}	5	0	11	0
n-Hexane	4.1	1.4×10^{-4}	90	82	80	25
n-Octane	5.2	6.3×10^{-6}	99	95	100	80

[a] Data from Schwarzenbach, et al. (1993).

Stability of epoxide hydrolase

Inactivation of epoxide hydrolase could be described by a first-order process. There was no significant difference between the fitted first order inactivation constants found for enzyme incubated in buffer and enzyme incubated in buffer saturated with octane (Fig. 2.2). The inactivation rate was 6-fold higher when a free interface was present in the Lewis cell, suggesting that, for octane, interfacial toxicity plays a greater role in the inactivation of epoxide hydrolase than molecular toxicity. The effect of shear on enzyme activity caused by the mixing at up to 200 rpm in the Lewis cell was tested and no significant inactivation was observed (data not shown). These results suggest that inactivation may be considerable when much higher interfacial areas are used.

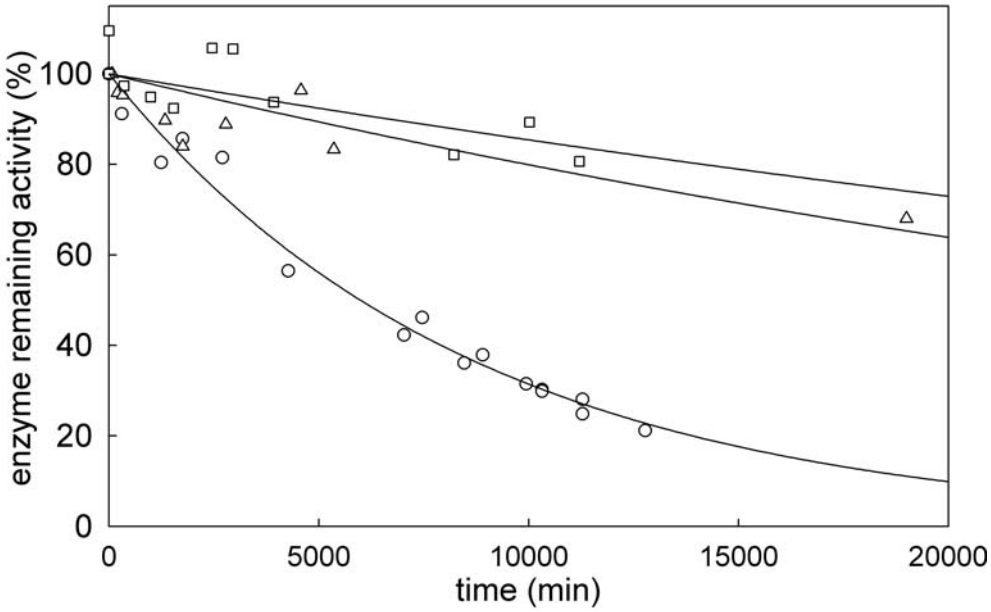

Figure 2.2. Inactivation of epoxide hydrolase. Enzyme solutions (0.11μM) were incubated in buffer (△) (k_d = 3.5x10^{-7} ± 1.7x10^{-7} s^{-1}); in buffer saturated with octane but with no separate octane phase (□) (k_d = 2.6x10^{-7} ± 0.9x10^{-7} s^{-1}); and in contact with octane in the Lewis cell (150 rpm) with an interfacial contact area of 22 cm^2 (○) (k_d = 1.9x10^{-6} ± 0.1x10^{-6} s^{-1}); all at pH 7.5 and 30^{o}C. The lines shown are exponential fits of the data.

Interphase mass transfer and partitioning of substrate and product

The transfer of substrate from the organic phase to the aqueous phase was followed in the absence of enzymatic reaction at 30 ^{o}C, using various concentrations of diol initially present in the aqueous phase. The value of k_a of 2.3x10^{-5}±0.3x10^{-5} m s^{-1}, with no diol present, did not vary significantly with increasing concentration of diol in the aqueous phase, but the partition coefficient, m, decreased with increasing diol concentration. In the range of

diol concentrations tested, where $D_S + D_R \leq 50\,\text{mM}$, the variation in m can be taken into account with a linear correlation:

$$m = 44.2 - 0.24(D_S + D_R). \qquad (2.14)$$

This means that as the reaction proceeds and diol accumulates in the aqueous phase, the flux of epoxide to the aqueous phase would increase in accordance with Eqs. 2.3 and 2.4.

From the equilibration experiments, the partition coefficient of diol over the two phases (organic/aqueous) was calculated as 0.03. Because of this low value, the transfer of diol to the organic phase was not taken into account in the model.

Kinetic resolution of styrene oxide in the Lewis Cell

Resolution experiments at various initial enzyme and epoxide concentrations were carried out in the Lewis cell to validate the mathematical model. Experimental conditions and model parameters are summarized in Table 2.3. The enantiomeric excess is a measure of the enantiomeric purity and is defined as $e.e. = (S - R)/(S + R)$.

Table 2.3. Experimental conditions and modeling parameters of resolution experiments shown in Figures 2.3 to 2.6.

Parameter	Figure 2.3A	Figure 2.3B	Figure 2.4	Figure 2.6
Initial styrene oxide conc. (mM)	20	20	126	324
$[E]_0$ (μM)	0.022	0.16	0.054	0.40
(U ml^{-1})	0.019	0.14	0.047	0.35
k_d (s^{-1})	5.6×10^{-6}	5.6×10^{-6}	7.1×10^{-6}	9.5×10^{-6}
k_a (m s^{-1})	2.3×10^{-5}	2.3×10^{-5}	2.3×10^{-5}	2.3×10^{-5}
A (m^2)	2.2×10^{-3}	2.2×10^{-3}	2.2×10^{-3}	4.5×10^{-2}
Total liquid volume (ml)	194	194	194	150
Φ	0.5	0.5	0.5	0.2

For an initial epoxide concentration of 20 mM, resolution of up to 37% e.e. was achieved with an enzyme concentration of 0.022 μM (Fig. 2.3A). The reaction was terminated before complete conversion had occurred because of the long reaction time. Use of the first-order rate constant found for enzyme inactivation determined with the Lewis cell did not yield satisfactory simulations of the obtained data. The simulation curves shown are obtained by fitting the inactivation constant, k_d. The fitted value of k_d (5.6×10^{-6} s^{-1}) is approximately three times higher than the k_d obtained in the inactivation measurements where no reaction takes place.

When a higher enzyme concentration (0.16 μM) was used (Fig. 2.3B), resolution was poor even though epoxide conversion occurred. The

concentration of both enantiomers in the octane phase decreased to 3 mM, after which the reaction was terminated due to the long reaction time. Using a k_d = 5.6x10^{-6} s^{-1}, simulated concentrations lie only slightly above the measured ones and the absence of resolution is predicted accurately, with e.e. remaining below 4%.

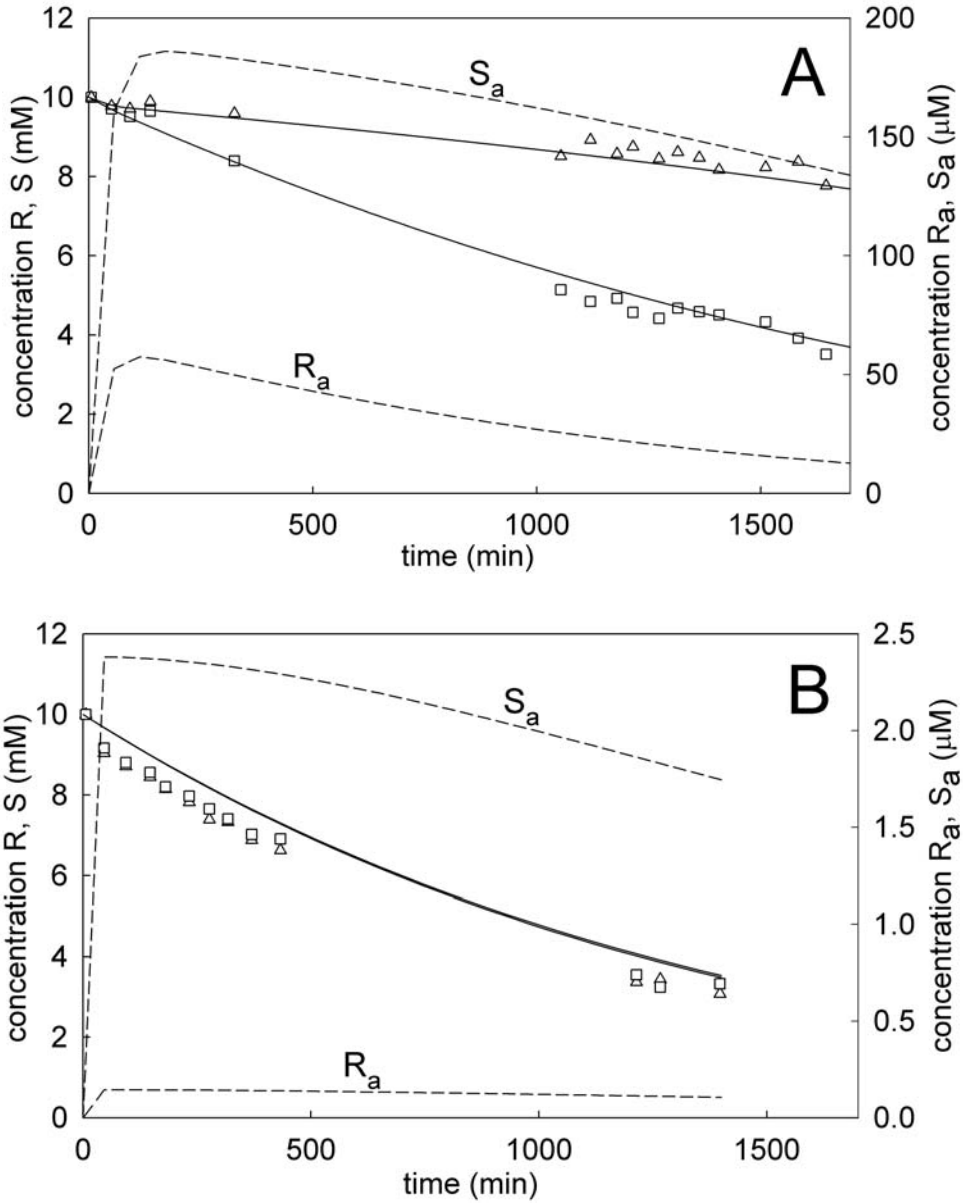

Figure 2.3. Resolution of styrene oxide in the Lewis cell. Profiles of (*R*) (□) and (*S*) (△) enantiomer conversion at different initial enzyme concentrations in the Lewis cell: (A) $[E]_0$ = 0.022 μM, (B) $[E]_0$ = 0.16 μM. Initial racemic styrene oxide concentration in the organic phase was 20 mM in both cases. Symbols indicate experimental data. Lines show model simulations of both the organic (full lines) and aqueous (broken lines) phase concentrations.

The enantioselectivity of the conversion of styrene oxide by this epoxide hydrolase is primarily due to the difference in the K_m values for the two enantiomers (Table 2.1) which results in the sequential conversion of the enantiomers. The (*S*)-enantiomer, which is converted second, also has a higher k_{cat} value and is hydrolyzed faster than the (*R*)-enantiomer. The poor resolution in this case can be explained by the fact that at the higher enzyme concentration the rate of mass transfer is slow compared to that of reaction, resulting in low aqueous phase concentrations of both enantiomers. The simulations show that the aqueous phase concentration of the (*R*)-enantiomer is below K_m^R (Fig. 2.3B) and therefore the (*S*)-enantiomer is also converted, thus reducing the effective enantioselectivity of the conversion. In the biphasic system it is important, therefore, to maintain sufficiently high concentrations of the (*R*)-enantiomer in the aqueous phase to inhibit the conversion of the (*S*)-enantiomer.

A resolution was also tested at an initial epoxide concentration of 126 mM (Fig. 2.4). The (*R*) and (*S*)-enantiomer concentrations in the organic phase decreased to 29 mM and 53 mM, respectively, corresponding to an e.e. of 28%, after which the reaction was terminated. After 4,000 min the concentrations of the two enantiomers and therefore also the e.e. started to become constant, deviating from the simulated curves calculated with k_d = 5.6×10^{-6} s^{-1}. This indicates that enzyme inactivation occurred at a faster rate than expected. The data was fitted to obtain a larger first-order inactivation constant, $k_d = 7.1 \times 10^{-6}$ s^{-1}.

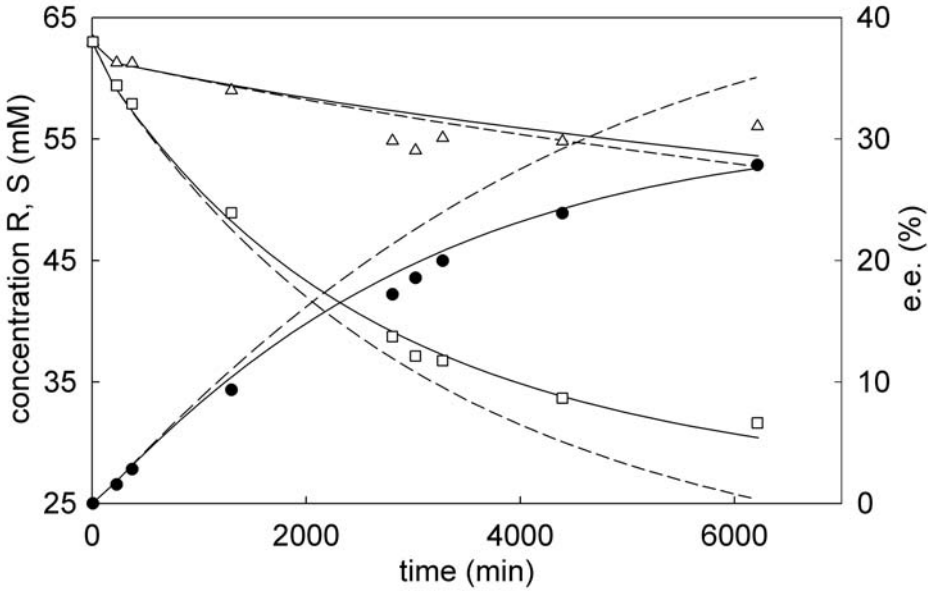

Figure 2.4. Resolution of styrene oxide in the Lewis cell. The initial racemic styrene oxide concentration in the organic phase was 126 mM. Initial enzyme concentration in the aqueous phase was 0.054 μM. Symbols indicate experimental data (S = △, R = □, e.e. = ●). Simulation lines are shown for two different values of first order inactivation rate constant: $k_d = 5.6 \times 10^{-6}$ s^{-1} (dotted lines), $k_d = 7.1 \times 10^{-6}$ s^{-1} (full lines).

With fitted values of k_d, there is good agreement between experimental data and model simulations. In all three experiments fitted values for k_d were higher than the value measured in the Lewis cell when no reaction takes place. Epoxides are very reactive compounds and toxic effects of epoxides on mono-oxygenases, used in alkene epoxidation, have been reported (Habets-Crutzen and de Bont, 1985; Prichanont et al., 1998; Stanley et al., 1992) although the specific mechanism for inactivation is still unknown. The higher enzyme inactivation rates found under reaction conditions for the resolutions may be caused by substrate toxicity to epoxide hydrolase and it is possible that this effect depends on the epoxide concentration. This is supported by the observation that when pure styrene oxide is used as the second phase, epoxide hydrolase is inactivated quickly and conversion stops before a reasonable enantiomeric excess is obtained (results not shown). Further studies were carried out to determine the effect of the diol product on the activity and stability of epoxide hydrolase and are described in Chapter 4.

Maximizing the yield of pure enantiomer

As discussed, a high enough concentration of the (*R*)-enantiomer must be present in the aqueous phase to inhibit the conversion of the (*S*)-enantiomer. The concentration of both enantiomers in the aqueous phase is dependent on the relative rates of mass transfer, enzymatic reaction and enzyme inactivation. Given that for a kinetic resolution the maximum attainable yield of pure enantiomer is 50%, the yield of (*S*) at time t, defined as:

$$Y_S = \frac{S_t}{S_0 + R_0}, \tag{2.15}$$

has to be maximized for highest process profitability. Process times should also be as short as possible to maximize space-time yield and to limit the proportion of epoxide converted by non-enantioselective chemical hydrolysis. This latter effect is most important when the rates of enzymatic hydrolysis and spontaneous hydrolysis are comparable, which at this operating pH occurs when enzyme concentrations are in the range of a few nanomolar. Low initial enzyme concentrations and enzyme inactivation also result in longer process times (for example, Fig. 2.4), in which a higher proportion of substrate is converted by spontaneous hydrolysis.

Based on the framework provided by Ramelmeier and Blanch (1989) for analyzing reaction regimes in two-liquid-phase systems, we derive an equation to determine the maximum enzyme concentration that should be used under certain mixing conditions, for which mass transfer limitations are sufficiently small, to achieve a good resolution in the shortest possible process time. We assume that mass transfer and reaction are processes in series. This assumption is justified since for all resolutions carried out in the Lewis cell, the Thiele modulus, ϕ, which gives information on the ratio of

the kinetic rate of reaction relative to the rate of mass transfer, is much less than 0.3. The Thiele modulus is defined as $\phi = \sqrt{\frac{k'[E]D}{k_a^{\,2}}}$ (Westerterp et al., 1993) where k' is an apparent enzymatic reaction rate constant defined as:

$$k' = \frac{k_{cat}^{S}}{S_a + \left(\frac{R_a}{K_m^R} + \frac{D_S}{K_i^{D_S}} + \frac{D_R}{K_i^{D_R}} + 1\right) K_m^S}. \tag{2.16}$$

Assuming a pseudo-steady state with $dS_a/dt = 0$, and substituting J_S from Eq. (2.3) and S_i from Eq. (2.1) in the mass balance over the aqueous phase (Eq. (2.10)),

$$k_a\left(\frac{S}{m} - S_a\right)\frac{a}{(1-\Phi)} = -r_S. \tag{2.17}$$

By rearrangement and using the reaction rate constant k' defined in Eq. (2.16) we obtain:

$$\frac{\left(\frac{S}{m} - S_a\right)}{\left(\frac{S}{m}\right)} = \frac{(S_i - S_a)}{S_i} = \frac{(k'[E] + k_c)S_a}{k_a\left(\frac{a}{1-\Phi}\right)}\frac{1}{S/m}, \tag{2.18}$$

This equation describes the dependency of the aqueous phase substrate concentration gradient over the film for mass transfer on the operating parameters which determine the rates of mass transfer and reaction. If there are no mass transfer limitations then the aqueous phase will be in equilibrium with the organic phase and $(S_i - S_a)/S_i = 0$. We assume that mass transfer limitations are sufficiently small when,

$$\frac{S_i - S_a}{S_i} < 0.1. \tag{2.19}$$

If the operating parameters k_a, a and Φ, are fixed, then the enzyme concentration [E] for which mass transfer limitations will cause only a small reduction in yield, can be calculated from this equation. Due to enzyme inactivation the active enzyme concentration decreases during the course of the reaction. It follows that the predicted maximum enzyme concentration, obtainable from Eq. (2.18), is a "lower limit value" so that the actual enzyme concentration which may be used will be slightly higher. It is clear from Eq. (2.18) that when enantioselectivity is almost absolute, in the case that $k_{cat}^S \sim 0$, or K_m^S is very high, so that k' is small, mass transfer limitations have a limited effect on decreasing the yield of pure (*S*)-enantiomer.

Resolution of styrene oxide in a batch emulsion reactor

Using the mathematical model, simulations were made to study the effect of varying enzyme concentration and specific interfacial area on Y_S at 95% e.e. and the process time required to reach 95% e.e. (Fig. 2.5). The influence of the value of k_d was also studied (for a = 300 m^{-1}). As expected, process times decrease with increasing enzyme concentration. When the mass transfer rate is low (a = 300 m^{-1}) the yield of (*S*) decreases rapidly when the enzyme concentration is increased. At an enzyme concentration of 1.4 μM, the predicted yield is close to zero. If no inactivation were to take place, a lower range of enzyme concentrations could be used to carry out a resolution since sufficient active enzyme would initially be present to reach the desired conversion. When k_d has the value $9.5x10^{-6}$ s^{-1} the lowest enzyme concentration which catalyses conversion to 95% e.e. is 0.1 μM. With a much higher mass transfer rate (a = 1000 m^{-1}), however, mass transfer limitations become important only for very high enzyme concentrations and low process times can be achieved with a relatively low decrease in Y_S. The simulations thus predict that the maximum enzyme concentration which can be used to obtain a good resolution is higher with a higher interfacial contact area.

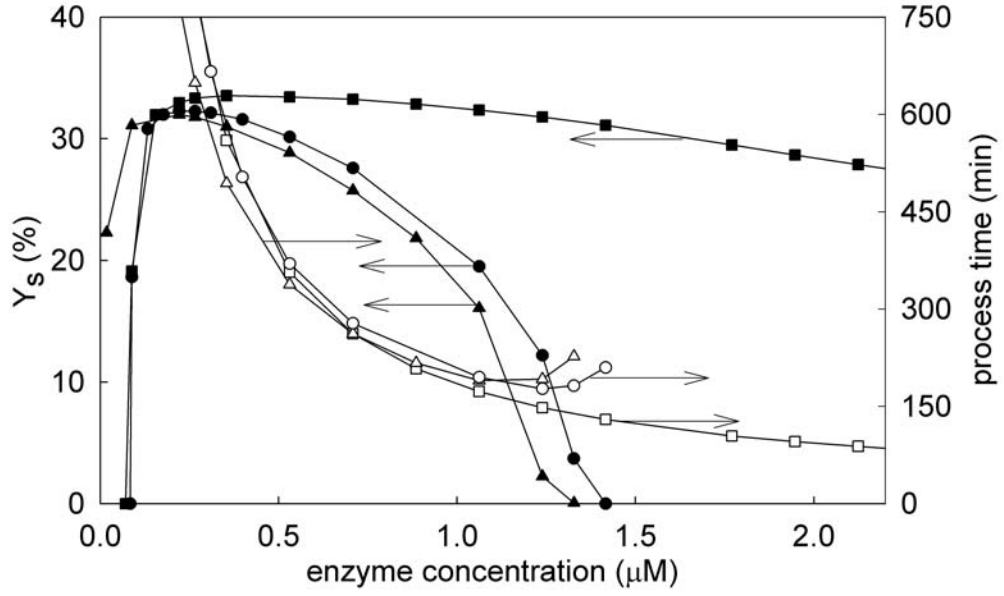

Figure 2.5. Effect of process parameters on resolution in an emulsion system. Simulations of the effect of enzyme concentration ([E]) and specific interfacial area (a) on the yield of pure (*S*)-enantiomer (Y_s) (closed symbols) at 95% e.e. and the process time required to reach 95% e.e. (open symbols): a = 1000 m^{-1} (■, □) for k_d = $9.5x10^{-6}$ s^{-1}. For a = 300 m^{-1}, two cases are shown: k_d = $9.5x10^{-6}$ s^{-1} (●, ○) and k_d = 0 (▲, △); For all the simulations k_a = $2.3x10^{-5}$ ms^{-1}; S_0 = R_0 = 162 mM; Φ= 0.2; total volume = 150 ml.

Based on these predictions, and to test the validity of Eq. (2.18), we tested the effect of a higher mass transfer rate with respect to operation in the Lewis cell by working with an emulsion where a larger interfacial area can be achieved. In Eq. (2.18) k' is a function of substrate and product concentrations, which vary during conversion. A simplification can be made by considering the relative magnitudes of substrate and product concentrations with respect to the Michaelis-Menten parameters (Table 2.1). D_S and D_R are of the same order of magnitude as the respective K_i values and will not influence k' to a great extent. Under good resolution conditions, when conversion of (S) is still low, $S_a >> K_m^S$ and when $R_a << K_m^R$, $k' = k_{cat}^S / S_a$, and since $k_c << k'$, k_c can be eliminated so that Eqs. (2.18) and (2.19) simplify to,

$$\frac{k_{cat}^S [E]}{k_a \left(\frac{a}{1-\Phi} \right)} \frac{1}{S_i} < 0.1. \qquad (2.20)$$

In the emulsion system tested, with $\Phi = 0.2$ and stirring at 200 rpm, the visually observed droplet diameters of approximately 3-5 mm corresponded to a specific interfacial area of 250-400 m^{-1}, but for calculation the specific interfacial area was fixed at 300 m^{-1}. The initial epoxide concentration in the organic phase was 324 mM, and using the criterion from Eq. (2.20) (with S_i = 3.7 mM), the maximum enzyme concentration which should be used to minimize the effect of mass transfer limitations is 0.30 μM. The uncertainty in the droplet diameter means that this calculated enzyme concentration could vary between 0.26 and 0.41 μM. The resolution was performed in such an emulsion system with an initial enzyme concentration $[E]_0$ = 0.40 μM (Fig. 2.6). Using this higher enzyme concentration, a good resolution could still be obtained since the active enzyme concentration decreased during the course of the reaction due to inactivation. From the experimental data, the enzyme first-order inactivation constant was fitted to $k_d = 9.5 \times 10^{-6}$ s^{-1}. The average active enzyme concentration over the course of the reaction, when the initial enzyme concentration is 0.40 μM, can be calculated as 0.34 μM using the approximation, $\frac{\langle [E] \rangle}{[E]_0} = \frac{1}{0.6 k_d t_{95\%} + 1}$ (Wolff et al., 1999).

For the conditions in this experiment, the model predicts a yield, Y_S, of 31.1% and a process time of 575 min (Fig. 2.5, for $k_d = 0$, $a = 300$ m^{-1} and [E] = 0.34 μM). There is good agreement between experimental data and model predictions and the uncertainty in specific interfacial area is of small importance since simulations show that Y_S, at 95% e.e., is relatively constant at 30 % for enzyme concentrations ranging from 0.26 μM to 0.41

μM, even for a = 250 m^{-1}. After 530 min, the e.e. reaches 96% with an analytical yield of (*S*) of 29.7%.

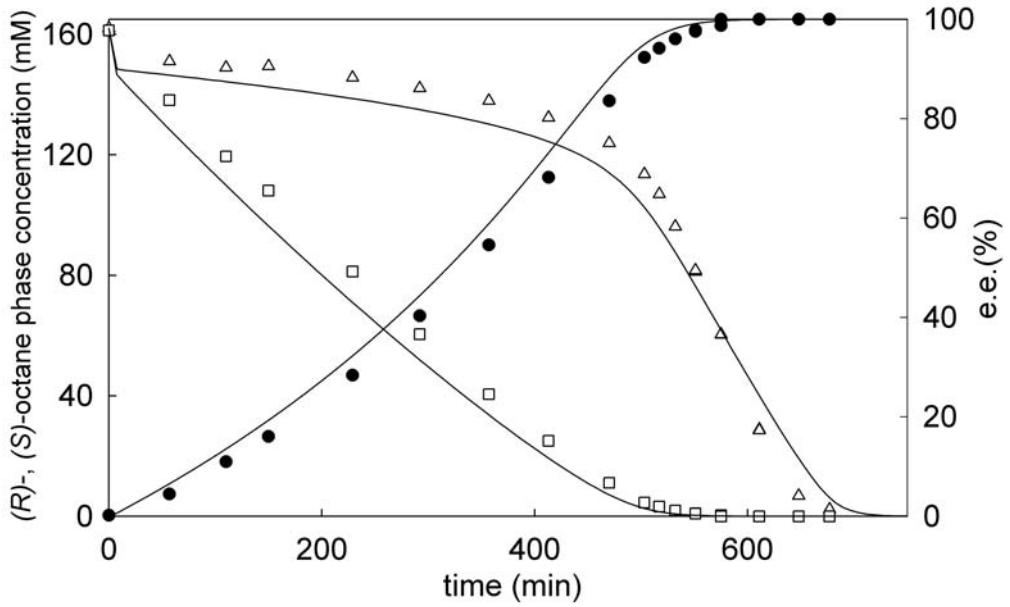

Figure 2.6. Resolution of styrene oxide in the batch emulsion reactor. The resolution was carried out in a total volume of 150 ml with Φ= 0.2 stirring at 200 rpm, for which a = 300 m^{-1}. Styrene oxide was fed to the organic phase at 324 mM. The initial enzyme concentration was 0.40 μM. Symbols indicate experimental data (R = □, S = △, e.e. = ●). Lines show simulation with a fitted first order inactivation constant k_d = 9.5x10^{-6} s^{-1}. At 96% e.e. the yield of (*S*)-enantiomer was 30%.

The conversion curve obtained (Fig. 2.7) was fitted to the equation derived by Chen et al. (1982) which relates e.e., conversion and E, to obtain an effective E value of 10. This is an improvement over the conversion in the Lewis cell of Figure 2.3A for which the effective E value was 3, but it is still lower than the intrinsic E value for the conversion of this substrate, of 16 (Fig. 2.7), suggesting that some mass transfer limitations still occurred under the chosen conditions. This may true at the beginning of the reaction, when transfer of substrate from the organic phase starts, and when enzyme activity is highest, and when most of the (*R*)-enantiomer has reacted, so that (*R*)-enantiomer aqueous phase concentrations are not high enough to inhibit conversion of the (*S*)-enantiomer. Furthermore, the intrinsic E value of 16 was determined with the kinetic parameters and does not take into account the effect of spontaneous hydrolysis. The measured E value of a resolution of styrene oxide in a single aqueous phase system at pH 9, including spontaneous hydrolysis, was 13.8 (Lutje Spelberg et al., 1998).

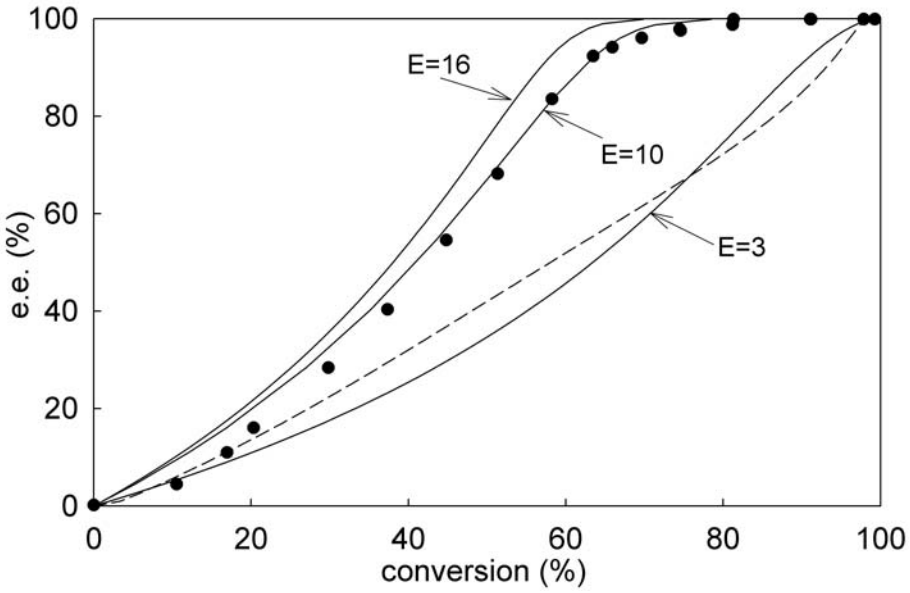

Figure 2.7. Comparison of resolution enantioselectivity in the Lewis cell and emulsion reactors. (dashed line) simulation of the resolution carried out in the Lewis cell (Fig. 2.3A); (●) experimental points of the resolution carried out in the emulsion reactor (Fig. 2.6). Fitted E values and corresponding curves are: E = 10 and E = 3 as shown. A theoretical curve for the intrinsic E value (16) is also shown.

A higher enzyme inactivation rate than that found for operation with a flat interface was expected due to the large increase in interfacial contact area. Studies on the inactivation of enzymes in liquid/liquid systems (Ghatorae et al., 1994) suggest that the amount of enzyme inactivated is proportional to the interfacial contact area. From the inactivation data (Fig. 2.2), it is clear that the presence of the interface causes part of the enzymatic inactivation. Since interfacial areas must be high enough so that mass transfer is not limiting, futher studies were carried out to determine the mechanism of interfacial inactivation of epoxide hydrolase in this biphasic system and are reported in *Chapter 3*.

Product inhibition becomes a limitation in this system only when diol concentrations exceed 1 M. This is because of the high diol inhibition constants relative to the affinity constants for both enantiomers of the substrate. To remove the diol formed during hydrolysis, ultrafiltration of the emulsion in a module external to the batch reactor could be carried out, recycling the organic phase and part of the aqueous phase with the enzyme back to the reactor. The applicability of this option has not been studied further here, but has recently been demonstrated for in-situ removal of an inhibitory product (Cauwenberg et al., 1999).

Conclusions

We have shown that the kinetic resolution of racemic epoxides by an enantioselective epoxide hydrolase can be carried out in an n-octane/aqueous two-phase system to overcome the problem of low epoxide aqueous solubility. A kinetic model was developed which could describe the bioconversion sufficiently accurately. Simulations showed that good resolution and high yields of enantiomerically pure epoxide can be obtained when mass transfer is not limiting. Based on the model, an equation for predicting the highest enzyme concentration which may be used to obtain a good resolution under defined mass transfer conditions and in a short process time was derived. This could be confirmed by a resolution of racemic styrene oxide at high concentration in a batch emulsion reactor, carried out under the predicted operating conditions. Agreement between predicted and observed yield of pure enantiomer was good.

Nomenclature

a	Specific interfacial area (interfacial area/total liquid volume), (m^{-1})
A	Interfacial contact area (m^2)
D	Diffusion coefficient ($m^2\ s^{-1}$)
D_S, D_R	(*S*) and (*R*) diol enantiomers, aqueous phase concentrations (mol m^{-3})
[E]	Enzyme concentration (mol m^{-3})
$\langle [E] \rangle$	Average enzyme concentration during a resolution (mol m^{-3})
E	Enantiomeric ratio $\left(\frac{k_{cat}^R / K_m^R}{k_{cat}^S / K_m^S} \right)$
J	Substrate flux (mol $m^{-2}\ s^{-1}$)
k_a	Mass transfer coefficient (m s^{-1})
k'	Apparent reaction rate constant ($m^3\ mol^{-1}\ s^{-1}$)
K_m^S, K_m^R	Michaelis-Menten parameters (mol m^{-3})
k_{cat}^S k_{cat}^R	Michaelis-Menten parameters (s^{-1})
K_i^{SD}, K_i^{RD}	Product inhibition constants for (*S*) and (*R*) diol (mol m^{-3})
k_c	Spontaneous hydrolysis kinetic constant (s^{-1})
k_d	First-order enzyme inactivation constant (s^{-1})
m	Epoxide partition coefficient (organic/aqueous)
S, R	(*S*) and (*R*) epoxide enantiomers, organic phase concentrations (mol m^{-3})

r_S , r_R	Rate of conversion of (*S*) and (*R*) enantiomers (mol m^{-3} s^{-1})
S_a , R_a	(*S*) and (*R*) epoxide enantiomers, aqueous phase concentrations (mol m^{-3})
S_i , R_i	(*S*) and (*R*) epoxide enantiomers, concentrations at the interface (aqueous side) in equilibrium with organic phase concentrations (mol m^{-3})
Y_S	Yield of (*S*)-enantiomer

Greek symbols

ϕ	Thiele modulus
Φ	Volume fraction of organic phase (organic phase volume/total volume)

Subscripts

0	at time = 0
t	at time = t

Chapter 3

Interfacial inactivation of epoxide hydrolase in a two-liquid-phase system

Abstract

Enantioselective epoxide hydrolases are useful biocatalysts for the preparation of enantiopure epoxides and diols. The kinetic resolution of racemic epoxides can be carried out in an organic/aqueous biphasic system to allow use of high epoxide concentrations. Enzyme inactivation in such a system, however, may occur by contact with the interface. In this study we investigated the factors which influence the interfacial inactivation of *Agrobacterium radiobacter* epoxide hydrolase in an octane/water biphasic system. Rates of interfacial inactivation were measured both in a stirred-cell, which has a plane interface, and an emulsion reactor. Interfacial inactivation rates measured in the stirred-cell at a fixed interfacial area increased with mixing intensity. Interfacial inactivation rates per unit area were lower in the emulsion reactor than in the stirred-cell and increased with bulk aqueous enzyme concentration. Circular dichroism analysis showed that during biphasic incubation all unadsorbed soluble enzyme existed in the native conformation. Activity assays showed that the dissolved enzyme was also fully active, indicating that inactivated enzyme precipitated from solution. Using an inactive epoxide hydrolase mutant structurally similar to the wild-type enzyme in order to avoid the conversion of the epoxide, it was found that high concentrations of epoxide in the organic phase increased the rate of interfacial inactivation.

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Introduction

Organic/aqueous biphasic mixtures can be used to increase the productivity of biocatalytic reactions when substrates are poorly soluble in water (Van Sonsbeek et al., 1993). The kinetic resolution of racemic epoxides by the epoxide hydrolase from *Agrobacterium radiobacter* AD1 can be successfully carried out to produce aromatic (*S*)-epoxides of high enantiomeric excess in a buffer/octane emulsion system. High epoxide organic phase concentrations can be used, although enzyme stability is not sufficient to permit reuse in successive batches (Baldascini et al., 2001). A preliminary analysis of enzyme stability in this system indicated that inactivation was mainly caused by direct contact of the enzyme with the octane-water interface, rather than by the interaction of dissolved octane and enzyme on a molecular level (Baldascini et al., 2001). Since large interfacial contact areas are required to ensure rapid mass transfer in the system, a detailed study of the factors that affect epoxide hydrolase interfacial inactivation under relevant operating conditions is required.

Enzyme susceptibility to interfacial inactivation is difficult to predict. Factors such as solvent polarity, interfacial tension (Ross et al., 2000a), aqueous phase pH (Kondo et al., 1991a) and ionic strength (Ross et al., 2000b; Kondo et al., 1991b) affect the extent of interfacial adsorption. The degree of enzyme structural rearrangement at an interface can be monitored with a variety of spectrophotometric techniques (Maste et al., 1997; Fang and Dalgleish, 1997; Corredig and Dalgleish, 1995) and is dependent on the solvent properties mentioned above, as well as surface load. Furthermore, properties of the protein such as secondary structure (Fang and Dalgleish, 1997), adiabatic compressibility (Ross et al., 2000), surface hydrophobicity, and thermostability (Ross et al., 2000b; Maste et al., 1997) are also correlated with the sensitivity to interfacial inactivation.

Interfacial inactivation can occur by enzyme adsorption to the interface followed by enzyme structural rearrangement (Fig. 3.1, steps 1 and 2) (Sadana, 1993, Norde and Giacomelli, 2000). Studies on protein interfacial adsorption and inactivation are frequently carried out under quiescent conditions or in discontinuous systems in order to determine adsorption kinetics and adsorption isotherms. However, the complete dynamic equilibrium which is established, including enzyme desorption from the interface (Fig. 3.1, step 3), should be considered for describing overall rates of enzyme interfacial inactivation in real biphasic reactor systems. In a liquid-liquid bubble column contactor, where the organic phase is bubbled through the aqueous phase, the enzyme solution is continuously exposed to a clean ('new') aqueous/organic interface to which no enzyme is initially adsorbed. For this type of contactor it has been shown that the amount of enzyme inactivated is proportional to the total interfacial area of solvent introduced (Ghatorae et al., 1994a; Ghatorae et al., 1994b). In

emulsion reactors, however, the rate of creation of 'new' interface, and thus the observed rate of enzyme inactivation, is not so well controlled. It is determined by the frequencies of droplet break-up and coalescence, which are a function of interfacial tension, liquid density difference, dispersed phase fraction, mixing intensity and reactor geometry (Wright and Ramkrishna, 1994; Walstra, 1993). Furthermore, the creation of 'new' interfacial area will also be affected by the rate of enzyme desorption from the interface if active enzyme in solution replaces the desorbed enzyme (Virkar et al., 1981), and this may be influenced by hydrodynamics.

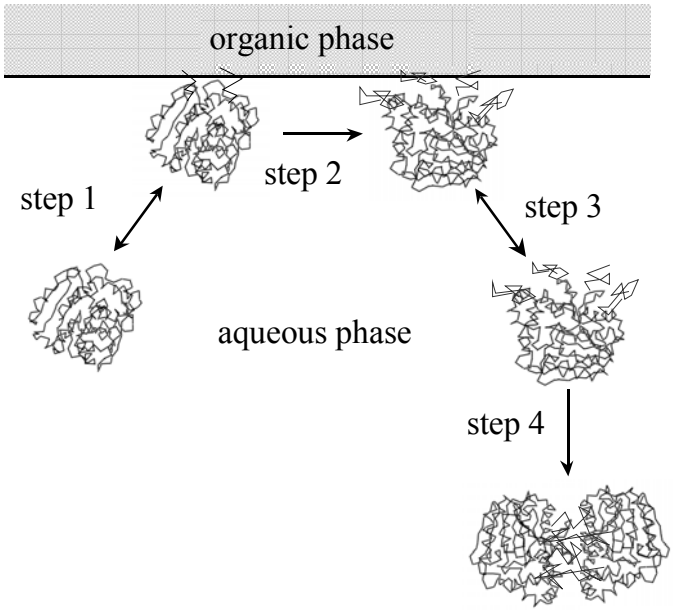


Figure 3.1. Schematic representation of the proposed mechanism of enzyme inactivation at an aqueous/organic interface. Step 1: reversible enzyme adsorption to the interface and concomitant enzyme structural rearrangement at the interface; step 2: further relatively fast unfolding of enzyme molecule at the interface; step 3: rate-limiting desorption of inactivated/unfolded enzyme molecules from the interface; step 4: irreversible aggregation and precipitation of inactivated enzyme.

In order to obtain more insight in the process of interfacial inactivation we have measured rates of inactivation of the epoxide hydrolase from *A. radiobacter* in biphasic incubations under various conditions. The effect of mixing intensity on the rate of enzyme interfacial inactivation was investigated using a stirred-cell contactor. In this set-up, a fixed plate with a circular opening between the two liquids stabilises and fixes the plane interface, allowing the mixing intensity in the aqueous phase to be varied independently of the interfacial contact area. Inactivation rates were also measured in an emulsion reactor under mixing conditions typically used for epoxide resolution experiments. Results showed that addition of epoxide to the organic phase increased enzyme interfacial inactivation.

Materials and methods

Chemicals and enzyme

Wild-type and mutant epoxide hydrolase preparations used in this work were produced as described by Rink et al. (1997). Purification was carried out with a DE52 anion exchange column. The partially purified enzyme was dialysed against TEMAG buffer (25 mM Tris-SO_4, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02 % sodium azide, and 10 % glycerol), concentrated to 14 mg ml^{-1} and stored at 4 ^oC. BSA powder (Fraction V, 96-99 % albumin) was obtained from Sigma.

p-Nitrostyrene oxide (pNSO) was synthesised as described elsewhere (Westkaemper and Hanzlik, 1981). N-octane (p.a. grade) was obtained from Fluka (Buchs, Switzerland) and was saturated with the aqueous phase buffer at 30^oC prior to use. The buffer composition in all experiments was 50 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5, unless otherwise stated.

Epoxide hydrolase activity assay

Enzyme activity assays were performed in a Perkin Elmer Lambda Bio 40 UV/VIS spectrophotometer with a temperature-controlled cell holder, by following the hydrolysis of the colorimetric substrate pNSO to its corresponding diol at 310 nm, for which extinction coefficients are ε_{310} = 4289 $M^{-1}cm^{-1}$ and ε_{310} = 3304 $M^{-1}cm^{-1}$, respectively. Typically, 1 ml of enzyme solution at an appropriate concentration was placed in a 1 cm quartz cuvette and the reaction was started by adding *(R)*-pNSO or racemic pNSO (dissolved in acetonitrile). The concentration of acetonitrile was kept below 1 % v/v since at high concentrations it is a competitive inhibitor of epoxide hydrolase. Conversion curves were either numerically fitted to the Michaelis-Menten equation to determine k_{cat} and K_m (for *(R)*-pNSO as substrate) using the software program Scientist (Micromath, Salt Lake City, UT) or used to obtain initial rates.

Enzyme stability in buffer

Stability of epoxide hydrolase was tested at 30 ^oC in 50 mM Tris buffer at pH 7.5 with additions of 1 mM β-mercaptoethanol and 1 mM EDTA, either separately or together. Stability was also measured with both additives at pH values of 7.5, 8, 8.5 and 9. For all incubations, enzyme activity was assayed periodically using racemic pNSO. Inactivation was described by a first order process. First order inactivation rate constants, k_d, were fitted using a least squares minimisation procedure, from which enzyme half-life values were calculated using $t_{1/2} = -\ln(0.5)/k_d$.

Stirred-cell contactor

A stirred-cell was used to measure rates of epoxide hydrolase inactivation in solutions contacted with octane while mixing at various stirring rates. The stirred-cell consisted of a thermostatted cylindrical vessel (7.5 cm diameter, 4.4 cm height) with two compartments separated by an interfacial plate. The bottom compartment was filled with 97 ml of enzyme buffer solution (0.0084 mg ml^{-1}) and an equal volume of octane was contained in the upper compartment. The interfacial plate was attached to four baffles (0.7 cm width) positioned at the edge of the plate near the vessel walls, and had a central hole (5.2 cm diameter, area 0.0022 m^2) to allow contact between the two phases. Both phases were stirred independently at equal rates (counter-current) by Rushton impellers (3.8 cm diameter, 0.7 cm blade width, 0.7cm blade height) mounted in the middle of the compartments.

Aqueous phase mass transfer coefficients, k_a, were determined by following the transfer of a model substrate, styrene oxide, from the organic phase to the aqueous phase in the stirred-cell at 30 ^oC, at stirring rates of 80, 150, and 190 rpm (stirrers rotating counter-currently at equal speeds in both phases). Values of the mass transfer coefficients and standard deviations were obtained by fitting the aqueous phase styrene oxide concentration profiles using a least squares minimisation procedure as described elsewhere (Baldascini et al., 2001).

Enzyme inactivation

Shear induced inactivation. The effect of shear due to mixing on enzyme stability was measured by incubating an aqueous enzyme solution (0.0093 mg ml^{-1}) in the stirred-cell reactor, kept at 30 ^oC, which was stirred with one Rushton turbine at rates of 200 and 300 rpm. The reactor was completely filled with the enzyme buffer solution to eliminate any air/water interfaces. Enzyme activity was determined periodically by the colorimetric assay already described. The stability of enzyme incubated in buffer, left unstirred, was measured for comparison.

Interfacial inactivation. Rates of inactivation of epoxide hydrolase incubated in the stirred-cell were measured to determine the effect of mixing rate on inactivation rate. Experiments were carried out at an enzyme concentration of 0.0084 mg ml^{-1}, at stirring rates of 50, 150 or 180 rpm. In each experiment stirring was increased carefully to the desired value to avoid breaking the flat interface. Periodically, 0.5 ml samples were taken from the aqueous phase, diluted by adding 0.7 ml of buffer, and remaining enzyme activity was determined by the colorimetric assay already described. The liquid removed during sampling was replaced by buffer in order to keep the interface at the height of the interfacial plate. The decrease in enzyme activity with time, after correction for dilution due to sampling, was

described by a first order rate constant, k_d, and values were obtained by fitting the data using a least squares minimisation procedure. Decrease in activity of a control incubation of enzyme in buffer (unstirred) was measured for comparison.

Enzyme inactivation rates were also measured in an octane/buffer emulsion system. The reactor used was the same cylindrical vessel of the stirred-cell containing four baffles but without the interfacial plate. An emulsion was created by adding both liquid phases to the cylindrical vessel and stirring with one Rushton impeller (dimensions as above) placed at half the total liquid height. The emulsion had a total volume of 150 ml with an organic volume phase ratio of 0.2, and was stirred at 200 rpm. This reactor set-up and operating conditions have previously been used in styrene oxide kinetic resolution experiments using a biphasic system (Baldascini et al., 2001). Inactivation rates were measured at initial enzyme concentrations of 0.0093 mg ml^{-1} and 0.1 mg ml^{-1}. All interfacial inactivation experiments were conducted at 30 ^oC.

Circular dichroism

Changes in epoxide hydrolase secondary structure were followed for a 0.1 mg ml^{-1} enzyme solution incubated in an emulsion system (at 30 ^oC) as described above. Far-UV CD spectra were recorded on an AVIV circular dichroism spectrometer (62ADS) by measuring the change in ellipticity in millidegrees. Samples of the aqueous phase were taken directly from the emulsion system at various times and spectra were recorded at 25 ^oC from 190-250 nm in a 1 mm cuvette. At later times during incubation, the samples were centrifuged at 13,000 rpm for 3 min to remove white precipitate-like particles prior to recording spectra. The spectra presented are the average of 3 scans using a bandwith of 1 nm, a step width of 1 nm and 5-s averaging per point. The spectra were corrected for buffer signal. As a control, CD spectra were also recorded for an unstirred enzyme solution (0.1 mg ml^{-1}) in buffer with no octane present, kept at 30 ^oC.

Effect of BSA addition on inactivation rate

The effect of addition of BSA to a buffer/octane emulsion on the rate of enzyme interfacial inactivation was tested. In 30 ml screw cap bottles, 14 ml of an epoxide hydrolase solution in buffer (0.013 mg ml^{-1}) were contacted with 6 ml of octane. The emulsions were kept at 30 ^oC and mixed with a 2 cm long magnetic stirrer at approximately 300 rpm. BSA was added to the aqueous phase (0.1 mg ml^{-1}) either together with epoxide hydrolase before mixing was started, or soon (5 min) after emulsification of octane with an epoxide hydrolase solution was started. Enzyme activity was measured periodically by the colorimetric assay.

Enzyme interfacial inactivation due to epoxide in octane

The effect of adding styrene oxide to the octane phase on the rate of interfacial inactivation was studied by following precipitation of an inactive epoxide hydrolase mutant (Y152F+Y215F) from an aqueous buffer solution which was vigorously mixed with octane containing various concentrations of styrene oxide. The screw cap bottle emulsion set-up described above was used for these experiments. Solutions of 0.1 mg ml^{-1} mutant epoxide hydrolase were contacted with octane containing either 0, 25, 250 or 600 mM styrene oxide. The emulsions were kept at 30 ^{o}C and mixed with a 2 cm long magnetic stirrer at 300 rpm. For comparison, the mutant enzyme was also incubated in buffer containing 0 or 6 mM (dissolved) styrene oxide (no octane) and stirred as the emulsion incubations. Periodically, soluble enzyme concentration was measured by the Bradford assay. Samples of 100 µl were taken from the aqueous phase and filtered by centrifugal filtration using ultrafree-MC microporous devices with a 0.2 µm pore size (Millipore BV), prior to addition to 1 ml Bradford reagent and measurement of adsorption at 595 nm.

Results and discussion

Enzyme stability in buffer

In order to investigate enzyme interfacial inactivation independently of other inactivation processes, we minimised enzyme inactivation in the aqueous phase by appropriate choice of pH and by adding mercaptoethanol and EDTA, compounds that are commonly used to stabilise enzymes (Schein, 1990). In 50 mM Tris buffer at pH 7.5 and 30 ^{o}C with no additives, the epoxide hydrolase had a half-life of 5.5 days. The half-life was increased to approximately 20 days by the addition of 1 mM EDTA and to 22 days by further addition of 1 mM mercaptoethanol. Optimum pH for enzyme stability was determined in buffer containing both the additives at pH values between 7.5 and 9. Enzyme stability was highest at pH 7.5, with a half-life approximately double that at pH 9. The selected aqueous phase composition for further experiments was 50 mM Tris, pH 7.5, 1 mM β-mercaptoethanol and 1 mM EDTA, where the enzyme showed remarkable stability with a half-life of approximately 24 days (k_d=1.95x10^{-5} ± 8.3x10^{-6} min^{-1}).

The effect of the shear forces created by mixing with the Rushton turbine on enzyme stability, in the absence of a liquid/liquid or air/liquid interface, was determined. Comparison with an unstirred enzyme solution showed that no significant shear-induced inactivation occurred over 150 h (data not shown). We conclude that in this reactor set-up shear-induced inactivation at an enzyme concentration of 0.0093 mg ml^{-1} and below a stirring rate of 300 rpm is neglectable.

Interfacial inactivation in the stirred-cell contactor

A stirred-cell was used to determine whether shear forces resulting from mixing acting at the interface influence the overall interfacial inactivation rate. This was done by measuring enzyme inactivation rates at stirring rates of 50, 150 and 180 rpm. Stirring rates were restricted to 50 - 190 rpm since above 190 rpm excessive rippling disturbed the interface. Below 190 rpm, the increase in interfacial area due to surface rippling was estimated to be less than 5%.

At the different stirring speeds, remaining enzyme activity was followed for 150 h. The enzyme activity decreased in time. The rate of enzyme inactivation decreased in time and could be described with a first-order inactivation rate constant, k_d. Enzyme inactivation rates increased with increasing stirring rate (Table 3.1, Fig. 3.2).

Table 3.1: Effect of different mixing conditions on interfacial inactivation of epoxide hydrolase. Enzyme was incubated in a stirred cell, using various stirring speeds, or in an emulsion reactor at different enzyme concentrations. Enzyme incubated in an unstirred buffer solution was used as a control. Inactivation was measured by following the remaining enzyme activity in time. k_d is the measured first order inactivation rate constant.

Incubation (reactor type)	Stirring rate (rpm)	Enzyme conc. (mg ml^{-1})	Aqueous phase volume (ml)	Total interfacial area (m^2)	k_d (x10^{-5} min^{-1})
Stirred-cell	50	0.0084	97	0.0022	3.4 ± 0.46
Stirred-cell	150	0.0084	97	0.0022	4.2 ± 0.39
Stirred-cell	180	0.0084	97	0.0022	6.4 ± 0.56
Emulsion	200	0.0093	120	0.045	9.9 ± 0.84
Emulsion	200	0.10	120	0.045	4.1 ± 0.36
Control	0	0.0084	-	0	1.0 ± 0.70
Control	0	0.10	-	0	0.56 ± 0.18

Although epoxide hydrolase inactivation by a molecular mechanism, where dissolved octane interferes with enzyme integrity, is minor compared to that which occurs by the interfacial mechanism (Baldascini et al., 2001), its contribution should be taken into account when comparing inactivation rates measured at different mixing rates. Assuming that the contributions of the molecular and interfacial mechanisms to the overall inactivation are additive, the first order rate constant for interfacial inactivation (k_i) was calculated by subtracting the first order inactivation rate constant for molecular inactivation (1.6×10^{-5} min^{-1} (Baldascini et al., 2001)) from the observed inactivation rate constant (k_d) in each experiment. Initial rates of interfacial inactivation per unit area of interface present in the system (initial

specific interfacial inactivation rate) were calculated to allow comparison between the different biphasic incubations. These were calculated by the formula, $\frac{k_i \times [E]_0 \times V_{aq}}{A_{\text{int}}}$, where $[E]_0$ is the initial enzyme concentration in the aqueous phase, V_{aq} is the aqueous phase volume, and A_{int} is the total interfacial area in the system. The initial specific interfacial inactivation rates were 6.6 x 10^{-3}, 9.6 x 10^{-3} and 1.8 x 10^{-2} mg min^{-1} m^{-2} for the stirring rates of 50, 150 and 180 rpm, respectively.

To account for the extent of inactivation observed in the experiments, an exchange between inactive enzyme molecules at the interface and active enzyme molecules from solution must take place. This is because, assuming that an epoxide hydrolase molecule is a sphere of 48 Å diameter (Nardini et al., 1999), a closely packed monolayer coverage of the interface would require a surface load of 3.1 mg m^{-2}. Thus, monolayer coverage requires only a small fraction of the total enzyme present. The increase in the observed inactivation rates with mixing intensity should be explained by considering the relative rates of enzyme adsorption, inactivation and desorption in the interfacial inactivation process.

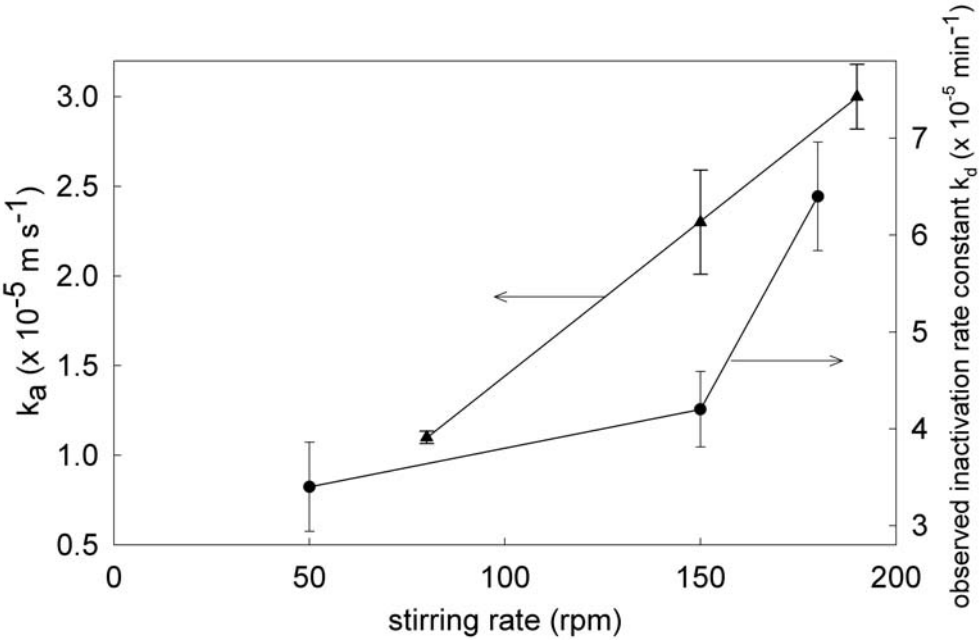


Figure 3.2. Interfacial epoxide hydrolase inactivation by contact with an octane/water interface in the stirred cell. Effect of stirring rate on (▲) the aqueous phase mass transfer coefficient k_a for transfer of styrene oxide from the organic to the aqueous phase, and on (●) the observed first order rate constant for epoxide hydrolase inactivation. Error bars show standard deviations obtained for fitting a single data set. The initial enzyme concentration was fixed at 0.0084 mg ml^{-1} and the interfacial area was fixed at 0.0022 m^2.

During the inactivation process, enzyme is first transported to the interface subsurface by convection and diffusion. At the interface, enzyme molecules adsorb and undergo a small structural perturbation (Fig. 3.1, step 1), possibly accompanied by loss of activity. This perturbation, and thus also adsorption, is reversible (Norde et al., 1986). The rate of enzyme adsorption is proportional to the aqueous enzyme concentration at the interface subsurface (Stuart, 1998). Adsorption rates of 36 mg min^{-1} m^{-2} have been found for proteins adsorbing to an air/liquid interface from an enzyme solution of 0.1 mg ml^{-1} (Ghatorae et al., 1994a).

Adsorbed enzyme molecules undergo further irreversible structural rearrangement (Fig. 3.1, step 2) due to attraction between the hydrophobic enzyme core and the hydrophobic interface (Sadana, 1993). Complete unfolding of the enzyme molecules may require long contact times (Fang and Dalgleish, 1997; Beverung et al., 1999), implying that a range of unfolded enzyme conformations can be present at the interface at any time. Lateral interactions between partially unfolded enzyme molecules adsorbed at interfaces may occur (Blank, 1969) and depend on surface coverage and extent of unfolding of the enzyme molecules. The extent of unfolding itself has been shown to decrease with increasing surface coverage (Fang and Dalgleish, 1997; Norde and Giacomelli, 2000; Roos and Walstra, 1996).

In step 3 unfolded/inactive enzyme desorbs from the interface. Whereas the adsorption step is recognised as a spontaneous process (Sadana, 1993), desorption of unfolded enzyme molecules from the interface does not occur spontaneously, for example by simple dilution of the aqueous phase, since it requires the disruption of the entropically favourable interactions between the enzyme and the solvent at the interface (Norde and Giacomelli, 2000; Norde at el., 1986; Stuart, 1998). Estimation of the rate of enzyme transport to the interface subsurface shows that it is not rate-limiting, therefore we suggest that the desorption of inactivated enzyme from the interface is the rate-limiting step in the inactivation process occurring in the stirred-cell contactor. At steady state, the interface is completely covered by native and unfolded enzyme, and the net rate of adsorption of native enzyme molecules from solution is equal to the rate of desorption of inactivated molecules. The increase in specific inactivation rate with stirring speed observed in these experiments would then suggest that increasing the mixing intensity increases the rate of desorption. The rippling effects observed at the interface were ascribed to motion of the interface perpendicular to the plane of the interface rather than expansion and contraction of the interface along the interfacial plane. The effect of this interface motion would be to increase the total interfacial area in the stirred cell, which was estimated as less than 5% at 180 rpm, rather than creation of ‘new interfacial’ area in time. Therefore the increased rate of interfacial inactivation cannot be explained by greater rippling motion at the interface. We conclude that the desorption of inactivated enzyme molecules from the interface is caused by

impingement of circulating eddies at the interface, whereby an increase in mixing intensity increases the rate of surface renewal and the magnitude of forces acting at the interface.

The aqueous phase mass transfer coefficient, k_a, for transfer of styrene oxide between the two phases was determined at the different stirring rates as a direct measure of mixing intensity in the aqueous phase. The k_a values increased linearly with increasing stirring rate (Fig. 3.2). The specific interfacial inactivation rates, however, increased more than proportionally with stirring speed. This different dependency on the stirring rate indicates that the rate of transfer of enzyme molecules to the interface subsurface is indeed not rate limiting, otherwise a linear increase in specific inactivation rate with stirring rate would also be expected. According to surface renewal theory of mass transfer, surface renewal rates at interfaces vary exponentially with mixing rates when the interface has a layer of adsorbed material because eddies require a certain momentum to clear an area at the interface (Davies, 1972). This suggests that the effectiveness of eddy-assisted enzyme desorption increases as the turbulence of mixing increases.

Interfacial inactivation in an octane/water emulsion

Successful enzymatic kinetic resolutions of epoxides in a biphasic system have been carried out in emulsions since high interfacial areas are required to ensure rapid interphase substrate mass transfer (Baldascini et al., 2001). Therefore, we also measured interfacial inactivation rates in an emulsion system typically used for these reactions. The total interfacial area in the emulsion set-up was calculated as 0.045 m^2 by visually estimating organic phase droplet sizes (0.4 cm diameter), corresponding to a 16-fold larger specific interfacial area compared to the stirred-cell set-up. The contribution of molecular inactivation due to dissolved octane to the total inactivation measured was taken into account as for the stirred-cell experiments. The initial specific interfacial inactivation rate for an enzyme aqueous phase concentration of 0.0093 mg ml^{-1} was calculated as 2.0×10^{-3} mg min^{-1} m^{-2}. This value is approximately 10 times lower than the highest specific inactivation rate found in the stirred-cell experiment operated at 180 rpm.

Comparison of the specific interfacial inactivation rates in the two systems shows that the rate of creation of ‘new’ interface to which enzyme from solution can adsorb is lower in the emulsion system than in the stirred-cell. In the emulsion system at steady state, ‘new’ interface is created by two mechanisms: by desorption of inactivated enzyme molecules from the interface, as also occurs in the stirred-cell, and additionally by the dynamic process of droplet coalescence and break-up since the surface which is created upon droplet break-up is initially free of adsorbed enzyme. The contribution of this latter mechanism to the creation of new interface in the

emulsion may in fact be low since enzyme adsorbed at the interface can hinder droplet coalescence (Walstra, 1993). Furthermore, the rate of desorption of inactivated enzyme molecules from the interface may also be lower in the emulsion compared to the stirred-cell since droplets freely follow flow patterns in the aqueous phase, possibly resulting in lower shear stresses at the interface. We conclude that emulsions with high interfacial areas can be exploited to obtain high solute interphase mass transfer rates with relatively low enzyme inactivation, since the interfacial inactivation rate is mostly determined by the rate of exchange between inactive and active enzyme molecules at the interface, rather than by the absolute amount of interface present in the system.

The effect of enzyme concentration on inactivation rate was investigated at two different values in the emulsion system (Table 3.1), at a fixed mixing rate. At an aqueous enzyme concentration of 0.1 mg ml^{-1}, the initial specific interfacial inactivation rate was calculated as $6.7x10^{-3}$ mg min^{-1} m^{-2}, which is approximately 3-fold higher than what was found at 0.0093 mg ml^{-1}. This value was calculated assuming that the first order rate constant for inactivation due to molecular toxicity of octane does not change with the bulk aqueous enzyme concentration, and is based on a total interfacial area of 0.045 m^2. The increase in specific interfacial inactivation rate with enzyme concentration suggests that the enzyme surface load at full interfacial coverage is higher when the bulk aqueous enzyme concentration is higher. This would be possible if the rate of adsorption (Fig. 3.1, step 1) increases relative to the rate of unfolding (Fig. 3.1, step 2), so that adsorbed and inactivated enzyme molecules exist in a more compact unfolded state at the interface. At the higher surface load, a larger number of inactive enzyme molecules would be desorbed per eddy clearance at the interface even though the rate of surface renewal remains constant since mixing rate is unchanged.

Epoxide hydrolase structural changes during interfacial inactivation

To further examine the loss of enzyme activity by interfacial contact, changes in enzyme structure during biphasic incubation were monitored by recording far-UV circular dichroism spectra of aqueous phase samples taken from an emulsion system at different points in time. Enzyme was present at an initial aqueous phase concentration of 0.1 mg ml^{-1}. In parallel, remaining enzyme activity was measured by the colorimetric assay.

Enzyme inactivation was accompanied by the formation of a white particulate precipitate in the aqueous phase. The decrease in soluble enzyme concentration in time, determined by the change in CD signal intensity at 220 nm, measured after sample centrifugation, indicated that the white particles were precipitated enzyme aggregates. Comparison of the shape of the far-UV CD spectra recorded from the emulsion incubation and from a

control incubation (no octane), after correction of signal intensity for the decrease in soluble enzyme concentration, showed that no change in the secondary structure of soluble enzyme took place over the whole period of incubation (Fig. 3.3). Therefore, all soluble unadsorbed enzyme had a native secondary structure.

Analysis of the kinetic data derived from the colorimetric activity assays showed that while the maximum rate of conversion of the substrate *(R)*-PNSO (U/ml of incubation mixture) decreased during the course of the incubation, the K_m of conversion remained the same. This also confirmed that no enzyme structural changes affecting the affinity of soluble enzyme for the substrate occurred during inactivation experiments. Furthermore, the decrease in soluble enzyme concentration correlated well with the measured decrease in enzyme activity (Fig. 3.4), from which it can be further concluded that all soluble enzyme remained catalytically active during biphasic incubation.

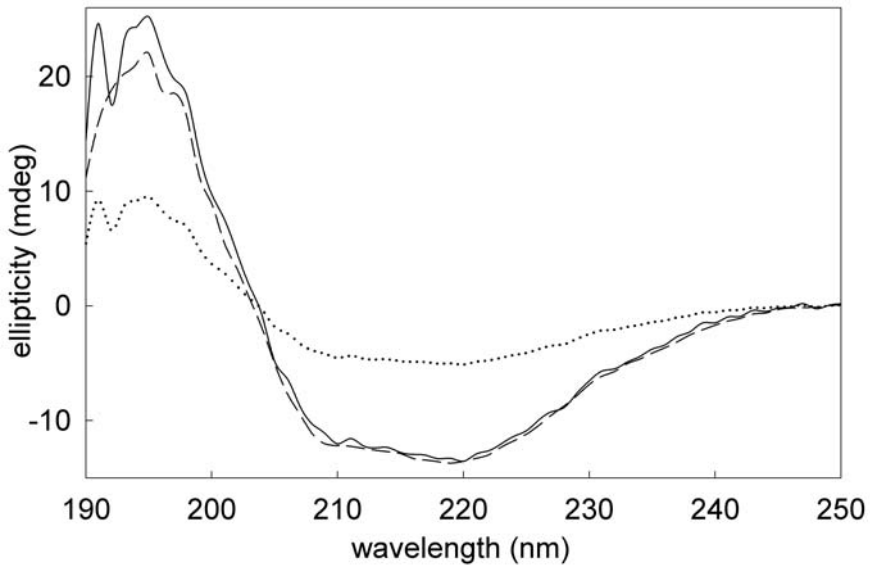


Figure 3.3. Effect of interfacial inactivation in an octane/buffer emulsion on the far-UV CD spectrum of epoxide hydrolase. The CD spectrum of enzyme incubated in an octane/buffer emulsion which was taken when enzyme activity had decreased by 65% after 359 hours of incubation (dotted line), was compared to the spectrum of enzyme incubated in only buffer for the same time (control) (dashed line). The CD spectrum of the sample taken from the emulsion system adjusted for the decrease in enzyme concentration is also shown (full line).

Since it is unlikely that unfolded enzyme molecules regain the native secondary structure after desorption from the hydrophobic liquid interface (Corredig and Dalgleish, 1995,Norde and Giacomelli, 2000), aggregation of inactivated enzyme molecules (by interaction of their hydrophobic cores)

with resultant precipitation from solution must occur before desorption or rapidly after desorption from the interface (Norde and Giacomelli, 2000) (Fig. 3.1, step 4) since no unfolded soluble enzyme was detected in the aqueous phase.

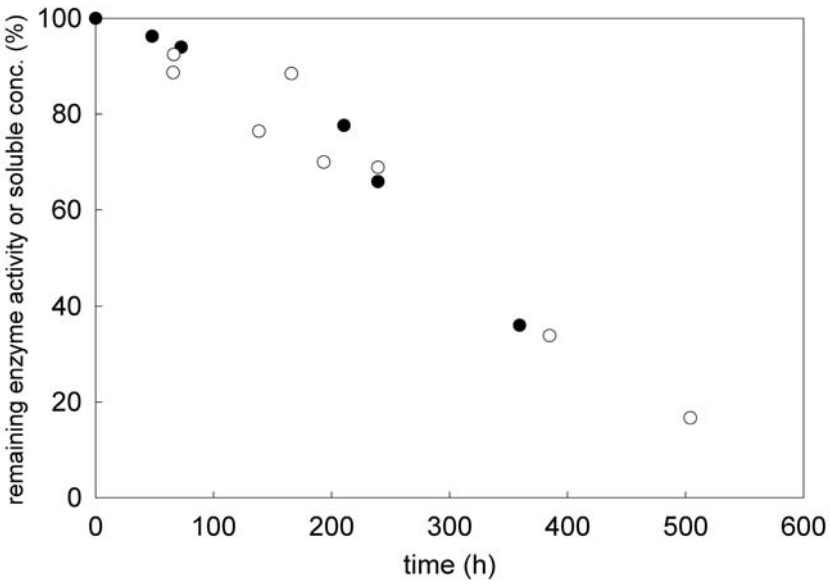


Figure 3.4. Inactivation of epoxide hydrolase in the emulsion system. Comparison of remaining enzyme activity (○) (relative to a control incubation with no octane) determined by the colorimetric assay; and (●) the remaining enzyme concentration in the aqueous phase (relative to a control incubation with no octane) determined by decrease in CD signal intensity at 220 nm.

Decreasing the rate of interfacial inactivation

Surfactants and macromolecular compounds have been widely tested for their ability to reduce protein interfacial inactivation. BSA is a protein frequently used for this purpose since it is highly surface-active (Beverung et al., 1999). Inactivation rates of epoxide hydrolase in a set of emulsion incubations were compared to determine whether addition of BSA could reduce the rate of epoxide hydrolase interfacial inactivation. The addition of BSA to the aqueous phase at a concentration of 0.1 mg ml^{-1} reduced the rate of interfacial inactivation of epoxide hydrolase considerably (Fig. 3.5). The order in which the two proteins were added and emulsification was initiated greatly influenced the degree of interfacial protection provided. When BSA was added after emulsification had taken place, its protective effect was much less than when BSA was added before emulsification was initiated. This indicates that the observed protecting effect was not due to a general stabilising property of BSA but suggests that the observed reduction in inactivation rate of epoxide hydrolase occurred as a result of the competition in adsorption of the two proteins at the interface.

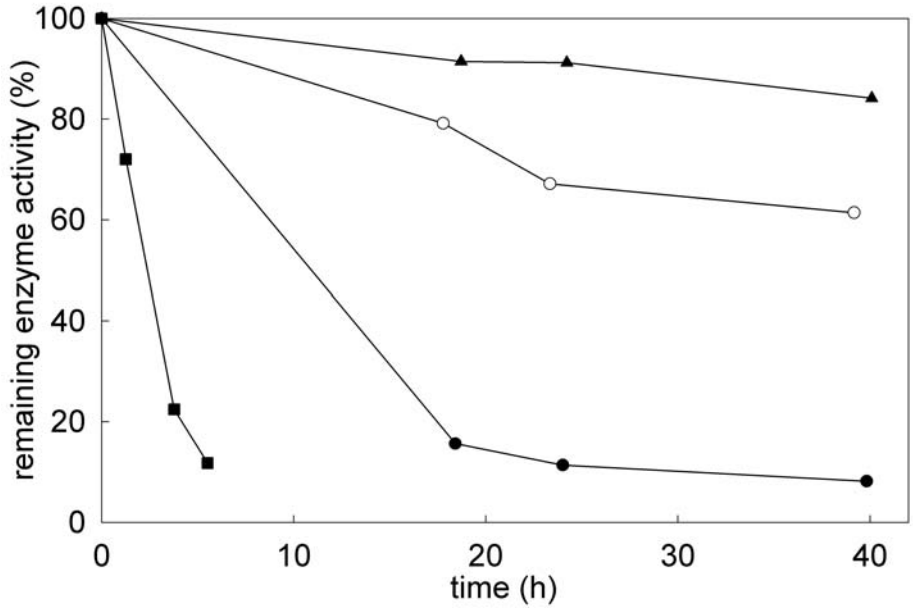


Figure 3.5. Effect of BSA addition on the rate of epoxide hydrolase inactivation incubated in octane/buffer emulsions. The remaining enzyme activity in time is given for: (▲) a control enzyme incubation (no octane), (■) an emulsion incubation with no BSA present, (●) an emulsion incubation in which BSA was added to the system 0.08 h after emulsification with epoxide hydrolase had been initiated, and (○) an emulsion incubation in which BSA and epoxide hydrolase were added together before emulsification was initiated. All incubations had a total volume of 20 ml and were stirred at 300 rpm with a 2 cm magnetic stirrer. Emulsions had an organic phase ratio of 0.3 v/v. The aqueous phase was composed of 50 mM Tris, 1 mM EDTA and 1 mM β-mercaptoethanol containing BSA and epoxide hydrolase at 0.1 and 0.013 mg ml^{-1}, respectively.

Effect of epoxide on interfacial inactivation

The experiments described above show that the rate of interfacial inactivation of epoxide hydrolase in an emulsion of pure octane and buffer is relatively low, and that at relevant enzyme concentrations enzyme half-life remains much higher than the reaction time of a typical batch biphasic kinetic resolution (10 h). However, the presence of epoxide substrate such as styrene oxide in octane at high concentrations, which is desirable for high process productivity, may cause an increase in the rate of enzyme inactivation (Baldascini et al., 2001). Using the wild-type epoxide hydrolase, the effect of epoxides on enzyme interfacial inactivation cannot be measured independently from stability effects arising from the formation of diol. Preliminary tests have shown that incubation of epoxide hydrolase in buffer containing 100 mM 1-phenyl-1,2-ethanediol, the hydrolysis product of styrene oxide, reduces enzyme half-life from 24 days to approximately 24 h. Therefore, to study the effect of the presence of styrene oxide in the organic

phase on epoxide hydrolase interfacial inactivation we used an inactive mutant of the epoxide hydrolase (Rink et al., 2000) in order to avoid the formation of diol during the experiment. The mutation of two tyrosine residues, normally situated in the active site, to phenylalanine (Y215F + Y152F) resulted in a six orders of magnitude decrease in k_{cat}/K_m values for conversion of styrene oxide, making the rate of diol formation by enzymatic conversion of the same magnitude as that by epoxide chemical hydrolysis. The concentration of diol formed during any incubation would be less than 7 mM, and tests have shown that this would cause negligible enzyme inactivation (data not shown). By CD spectroscopy, this mutant has been shown to have an identical overall structure to the wild-type enzyme (Rink et al., 2000), so we assume that it would undergo inactivation/unfolding upon adsorption by the same mechanism as wild-type enzyme. Using this mutant enzyme, interfacial inactivation rates in emulsions containing styrene oxide were measured by monitoring the decrease in dissolved enzyme concentration in time. This is justified since we have shown above that interfacial inactivation results in enzyme precipitation from solution.

The mutant enzyme was incubated in emulsions containing different styrene oxide concentrations in the octane phase. The emulsions were stirred with a magnetic stirrer giving organic phase droplets of approximately 0.4 cm diameter. Control incubations consisted of enzyme in aqueous buffer, with 0 or 6 mM dissolved styrene oxide, which were similarly stirred.

In all incubations, the soluble enzyme concentration decreased in time (Fig. 3.6). In the control incubation with no epoxide and no octane, stirring alone caused approximately 10 % enzyme precipitation over the 24 h incubation period. This may have been largely due to enzyme denaturation at the air/water interface (Virkar et al., 1981). Precipitation of epoxide hydrolase in an aqueous solution of 6 mM styrene oxide was tested to account for enzyme inactivation due to molecular toxicity of styrene oxide. In this incubation, 30 % enzyme precipitation occurred over the period of incubation (Fig. 3.6) which suggests that some precipitation can be caused by the molecular toxicity of styrene oxide. Nevertheless, enzyme inactivation in all biphasic incubations was faster than in the single aqueous phase incubation containing styrene oxide, indicating that inactivation in the biphasic systems was caused mainly by interfacial effects.

The presence of styrene oxide above a threshold concentration increased the rate of interfacial inactivation. At a styrene oxide octane phase concentration of 600 mM, the inactivation was considerably faster than at 250 mM. However, below 250 mM the rate of interfacial inactivation did not appear to change with styrene oxide concentration. The increase in interfacial inactivation rate could be due to a decrease in interfacial tension which is expected upon addition of styrene oxide. A lower interfacial tension would result in a lower average droplet diameter, and thus a greater steady state interfacial area. This could not be confirmed experimentally since the

droplet sizes could only be determined approximately. Why the effect is observed only above a threshold epoxide concentration remains unclear. A decrease in interfacial tension would in principle also increase the frequency of droplet coalescence and dispersion, resulting in a higher rate of creation of 'new' interface and thus a higher rate of interfacial inactivation.

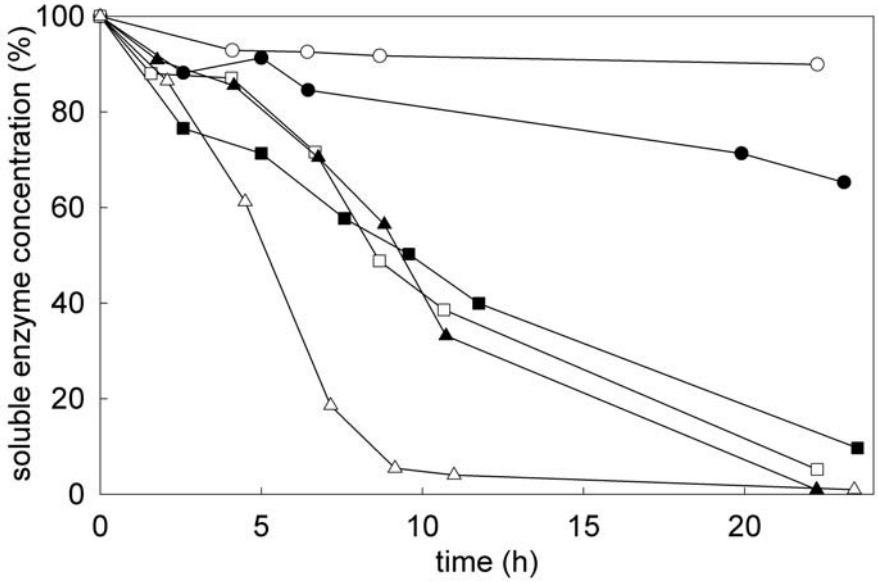


Figure 3.6. Effect of styrene oxide in the organic phase on the rate of epoxide hydrolase inactivation. 14 ml of aqueous solutions of an inactive epoxide hydrolase mutant (Y215F+Y152F) at 0.1 mg ml^{-1} were contacted with 6 ml octane containing styrene oxide at varying concentrations. Change in soluble enzyme concentration was measured at each time point by the Bradford assay. Styrene oxide concentrations: (△) 600 mM in octane, (▲) 250 mM in octane, (□) 25 mM in octane, (■) 0 mM in octane, (●) 6 mM aqueous phase (no octane), (○) 0 mM (no octane).

Conclusions

Inactivation of epoxide hydrolase at the octane/water interface takes place by unfolding of enzyme molecules adsorbed at the interface, followed by enzyme aggregation and finally precipitation from solution. Increasing the mixing intensity was found to increase the rate of interfacial inactivation and we propose that this effect is due to an increase in the rate of desorption of inactivated enzyme molecules from the interface which then allows active enzyme in solution to become adsorbed and inactivate in turn.

By comparing interfacial inactivation rates in a stirred cell and an emulsion system we have shown that the use of an emulsion system can be exploited to obtain high solute interphase mass transfer rates since the rate of specific interfacial inactivation remains low. However, in this system, the

presence of epoxide substrate at high concentration in the organic phase increases the rate of interfacial inactivation. Addition of a sacrificial protein to the system, which can prevent adsorption of the catalytic enzyme at the interface, could provide a method to reduce the rate of interfacial inactivation.

Chapter 4

Inactivation of epoxide hydrolase by vicinal diols

Abstract

Epoxide hydrolase from *Agrobacterium radiobacter* AD1 catalyses the enantioselective hydrolysis of epoxides to their diols. Since the feasibility of such biotransformations is largely determined by the possibility to accumulate product to high concentrations, we have investigated the effect of diols on epoxide hydrolase activity and stability. Stability experiments were conducted by measuring the change in enzyme activity in time for enzyme incubated in diol solutions. Diols caused a time-dependent loss of enzyme activity which was characterised by bi-exponential decay. The rates of inactivation increased with increasing diol concentrations. The inactivation was specific for vicinal diols, which also act as competitive inhibitors, and the inactivating effect was greater for diols that were also stronger inhibitors. Active site specificity was demonstrated by protection from inactivation with the competitive inhibitor phenyl acetamide. Inactivation did not result in enzyme aggregation and precipitation from solution but was nevertheless only partially reversible. Circular dichroism (CD) and fluorescence spectroscopy were used to identify changes in enzyme structure that occurred during inactivation. CD spectra recorded in the far-UV region demonstrated that the secondary structure of epoxide hydrolase remained unaltered during inactivation by 1,2-octanediol, whereas dramatic changes assigned to tyrosine and tryptophan residues occurred in the near-UV region. The active site tyrosine mutant (Y215F) was resistant to inactivation by vicinal diols indicating that the presence of the two tyrosines in the active site is necessary for inactivation. We propose that the inactivation occurs by a three step mechanism, comprising modification at the active site by either irreversible covalent binding of the diol to the enzyme or an irreversible local structural change.

Introduction

The kinetic resolution of racemic epoxides has emerged as an attractive route for industrial production of enantiomerically pure epoxides which are valuable fine chemical intermediates (Archer, 1997; Manoj et al., 2001). In order to achieve high process productivity, biotransformations must be operated at high substrate concentrations. However, the use of high epoxide concentrations has been shown to reduce biocatalyst activity and/or stability both for enantioselective epoxide hydrolysis reactions and epoxidation reactions (Krieg et al., 2001; Manoj et al., 2001; Morisseau et al., 1997; Prichanont et al., 1998). Several studies suggest that the enzyme inactivation observed in epoxidation reactions is caused by a chemical modification of the enzyme by the produced epoxide due to the high reactivity of the epoxide ring (Habets-Crützen and de Bont, 1985; Prichanont et al., 1998; Stanley et al., 1992). The decrease in enzyme activity during kinetic resolution of racemic epoxides, however, has generally been attributed to product inhibition by the diol (Choi et al., 1999; Genzel et al., 2001; Tang et al., 2001). The epoxide hydrolase from *Agrobacterium radiobacter* AD1 is a promising enzyme for application in enantioselective hydrolysis of epoxides in two-phase systems (Baldascini et al., 2001) and a suitable protein for mechanistic studies since the structure and kinetics of the enzyme have been studied in detail (Rink and Janssen 1998a; Rink et al., 2000; Nardini et al, 1999). Here we investigate the effect of diols on the activity and stability of this enzyme.

Vicinal diols are competitive inhibitors of this epoxide hydrolase (Baldascini et al., 2001). During the kinetic resolution of a racemic mixture, the reduction in reaction rate caused by product inhibition depends on the relative magnitudes of the diol inhibition constant, K_i, and the corresponding substrate affinity constant, K_m. The diols produced from hydrolysis of industrially relevant epoxides such as styrene oxide are relatively poor inhibitors of this enzyme, having K_i values in the millimolar range. Millimolar concentrations are therefore required to cause appreciable reduction of enzyme activity during hydrolysis (Baldascini et al., 2001) particularly since the corresponding substrates are converted with high affinity. Nevertheless, in industrial applications diols can easily accumulate to high concentrations so that the effect of inhibition on the course of reaction can be substantial.

Besides a reversible inhibitory effect, preliminary studies showed that incubation of this epoxide hydrolase in aqueous solutions containing diols at high concentrations could cause time-dependent loss of enzyme activity. In this chapter the extent and mechanism of diol-induced enzyme inactivation are investigated. We aimed to establish whether or not inactivation was caused by protein unfolding, to identify the specificity of inactivation with respect to diol structure, and to find ways of reducing the inactivation. To

this end the inhibitory and inactivating effects of structurally diverse diols on this epoxide hydrolase was quantified. Analysis of enzyme conformational changes occurring during inactivation was performed by circular dichroism (CD), steady state fluorescence spectroscopy and electrospray ionisation-mass spectrometry (ESI-MS). Spectroscopic analysis indicated that inactivation by 1,2-octanediol was accompanied by a local conformational change at the enzyme active site. Based on the kinetic and spectrophotometric data obtained, we propose that the inactivation occurs by a mechanism which includes rapid and reversible binding of the diol at the enzyme active site followed by a slow reversible inactivation step, and a further slow irreversible inactivation step that may involve covalent binding of the diol to the enzyme. Importantly, an active-site mutant (Tyr215Phe) was resistant to inactivation by vicinal diols.

Materials and methods

Chemicals and enzyme

Partially purified wild-type and mutant epoxide hydrolase preparations were used in this work. Enzyme was produced as described by Rink et al. (1997). Purification was carried out with a DE52 anion exchange column. The partially purified enzymes (minimum 75 % pure) were dialyzed against TEMAG buffer (25 mM Tris-SO_4, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, and 10% glycerol) and concentrated to approximately 14 mg ml^{-1} and stored at 4 ^oC.

Racemic, *(R)* and *(S)*-*p*-nitrostyrene oxide (pNSO) were synthesised as described elsewhere (Westkaemper and Hanzlik, 1981). *(R)* and *(S)*-1-phenyl-1,2-ethanediol (PED) (99%), racemic PED (97%), 3-phenoxy-1,2-propanediol (PPD) (95%), 3-chloro-1,2-propanediol (CPD), glycerol, 1,2-octanediol (98%), 1,2-hexanediol (98%), 1,2-butanediol (99%), 1,2-propanediol (99.5%) were obtained from Aldrich and 1,8-octanediol (99+%) was obtained from Acros. TEM buffer was of composition 50 mM Tris-SO_4, pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol.

Epoxide hydrolase activity assay

Enzyme activity assays were performed in a Perkin Elmer Lambda Bio 40 UV/VIS spectrophotometer at 30 ^oC, by following the hydrolysis of the colorimetric substrate pNSO to its corresponding diol at 310 nm. The corresponding extinction coefficients are ε_{310} = 4289 $M^{-1}cm^{-1}$ and ε_{310} = 3304 $M^{-1}cm^{-1}$ for the epoxide and diol, respectively. Typically, 1 ml of enzyme solution (diluted from an original sample to approx. 0.06 μM) was placed in a 1 cm quartz cuvette and the reaction was started by adding *(R)*, *(S)* or racemic pNSO (dissolved in acetonitrile) to an appropriate concentration. The concentration of acetonitrile was kept below 1% v/v

since at high concentrations it is a competitive inhibitor of epoxide hydrolase. Conversion curves were used to derive initial rates or were fitted to obtain k_{cat} and K_m values.

Inhibition of epoxide hydrolase

Competitive-inhibition constants for various diols were determined as described by Rink et al. (2000) by measuring the effect of different inhibitor concentrations on the conversion of *(S)*-pNSO by wild-type epoxide hydrolase under first order conditions, at 30 °C. Depletion curves were recorded with 50 μM *(S)*-pNSO and varying inhibitor concentrations. The apparent k_{cat}/K_M values, $(k_{cat}/K_M)^{app}$, were determined by fitting a single exponential to the recorded traces. The inhibition constant, K_i, was obtained by fitting the formula $(k_{cat}/K_M)^{app} = (k_{cat}/K_M)/(1+[I]/K_i)$ to the $(k_{cat}/K_M)^{app}$ values obtained at several inhibitor concentrations [I]. Inhibition constants for the mutant epoxide hydrolase (Y215F) were determined in a similar manner, using *(R)*-pNSO as the colorimetric substrate at a concentration of 50 μM for conversion under first order conditions. The K_i values reported have an error of ± 10%.

Effect of diols on enzyme stability

The effect of several diols on enzyme stability at 30 °C was measured by incubating epoxide hydrolase in diol solutions and periodically taking samples from the incubations to measure the remaining enzyme activity by the colorimetric assay already described. The remaining enzyme activity is reported as a percentage of the activity at time zero. At time zero, enzyme activity was measured with a sample taken from the enzyme incubation solution with diol already present at the desired concentration. Enzyme was incubated at a concentration of 0.005 mg ml^{-1}, in TEM buffer containing racemic 1-phenyl-1,2-ethanediol (PED), 3-phenoxy-1,2-propanediol (PPD), 3-chloro-1,2-propanediol (CPD) and glycerol at concentrations of 25, 50 and 100 mM. The effects of 1,2-hexanediol, 1,2-butanediol and 1,2-propanediol on enzyme stability were determined in TEM buffer solutions at diol concentrations of 100 mM. The effect of 1,2-octanediol was tested at a concentration of 25 mM since this was close to the solubility limit and 1,8-octanediol was also tested at 25 mM to enable direct comparison between the two isomers. Enzyme was incubated at a concentration of 0.01 mg ml^{-1}. The time course of inactivation of epoxide hydrolase by PED and PPD in the presence of phenylacetamide, a strong inhibitor of epoxide hydrolase, was tested by incubating enzyme at a concentration of 0.0077 mg ml^{-1} in 100 mM diol solutions containing phenylacetamide at different concentrations. Remaining enzyme activity was measured in time. Activity at time zero was measured with a sample from the mixture in which all components were already present. In all cases, remaining enzyme activity for enzyme incubated in TEM buffer was measured for comparison.

To determine whether the inactivation was reversible, diol was removed from inactivated samples by repeated dialysis against TEM buffer at 30 ^{o}C, over a period of 66 h. After dialysis the enzyme sample was kept at 30 ^{o}C and enzyme activity was measured periodically. For enzyme incubated with 100 mM PED, dialysis was carried out with samples taken after 40% and 90% activity had been lost to determine whether the extent of enzyme inactivation influenced the reversibility. In separate experiments, enzyme samples which had been inactivated by incubation in 100 mM PPD, 100 mM PED or 21 mM 1,2-octanediol were repeatedly dialyzed against TEM buffer at 4 ^{o}C for a period of 66 h and remaining enzyme activity of the recovered samples was measured.

CD and fluorescence measurements

Changes in steady-state fluorescence spectra of epoxide hydrolase incubated in diol solutions were monitored on an SLM Aminco SPF500-C spectrofluorometer. Enzyme was incubated in solutions of 100 mM PED, 25 mM 1,2-octanediol and 25 mM 1,8 octanediol at 30 ^{o}C and fluorescence spectra of samples were taken periodically. Prior to spectral analysis, precipitated enzyme was removed by centrifugal filtration with ultrafree-MC microporous devices with a 0.2 μm pore size (Millipore BV). Samples were excited at 290 nm and fluorescence emission was followed from 300 to 500 nm. Spectra were corrected for absorbance of the incubating solution. Dissolved enzyme concentration of incubated enzyme was measured in parallel by the Bradford assay. A solution of epoxide hydrolase incubated in TEM buffer was tested in a similar manner as a control.

Circular dichroism measurements were done on an AVIV circular dichroism spectrometer (62ADS). Far-UV spectra were recorded at 25 ^{o}C from 190-250 nm in a 0.1 cm quartz cuvette. The spectra presented are an average of three scans, each recorded using a bandwith of 1 nm, a step width of 1 nm and 5-s averaging per point, and were corrected for absorbance caused by the incubating solution. Spectra were taken for epoxide hydrolase incubated in 1,2-octanediol at the start of incubation and after 11 days. Far-UV CD spectra of enzyme inactivated by incubation in a 100 mM PPD solution were taken after removal of the diol by repeated dialysis and concentration of the enzyme solution to approximately 0.1 mg ml^{-1}.

Near-UV CD spectra of wild-type and mutant epoxide hydrolases incubated in TEM buffer at a concentration of 2 mg ml^{-1} were recorded at 25 ^{o}C. Changes in near-UV CD spectra of wild-type and Y215F mutant epoxide hydrolase incubated at a concentration of 2 mg ml^{-1} in 21 mM 1,2-octanediol were followed by taking spectra at different times during the incubation. Spectra were recorded from 250-310 nm using a 0.5 cm quartz cuvette. The spectra presented are an average of three scans, each recorded using a bandwith of 1 nm, a step width of 1 nm, and 5 -s averaging per point. The spectra were corrected for absorbance caused by the incubation solution and

any change in enzyme concentration which occurred during the incubations. Remaining enzyme activity was measured in parallel by the colorimetric assay and the soluble enzyme concentration was determined by the Bradford assay. Prior to spectral analysis any precipitated enzyme was removed by centrifugal filtration (0.2 μm) with ultrafree-MC microporous devices. As a control, enzyme incubated in TEM buffer was analyzed in the same way.

ESI-MS

The molecular mass of inactivated wild-type enzyme was determined by ESI-MS and compared to that of unmodified, active enzyme. Before analysis, the inactivated enzyme was dialyzed repeatedly against TEM buffer (pH 7.5) at 4 ^{o}C to remove any unbound diol, washed with ultrapure water containing 1% formic acid, and then dissolved in 80% acetonitrile containing 0.4% formic acid. ESI-MS was performed on an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada), a triple quadrupole mass spectrometer supplied with an atmospheric pressure ionization source, and an ionspray interface. The spectra were scanned in the range between *m/z* 400 and 1600.

Results

Time-dependent irreversible enzyme inactivation

The effect of diols on enzyme stability was tested by incubating purified *A. radiobacter* epoxide hydrolase in diol solutions and measuring remaining enzyme activity in time. Incubations were carried out with a range of diol concentrations that would typically be reached during large-scale kinetic resolutions of epoxides (25 - 100 mM). Inactivation of epoxide hydrolase over a period of hours was observed when the enzyme was incubated with PED, PPD which are the products of the hydrolysis of styrene oxide and phenylglycidylether, respectively (Table 4.1). No appreciable enzyme inactivation occurred in the presence of CPD, the product of the hydrolysis of epichlorohydrin. Incubation with 100 mM glycerol had a stabilising effect, indicating that a non-specific denaturing effect between the enzyme and the hydroxyl moieties of the diols was not the cause of the inactivation.

The rate of enzyme inactivation increased with increasing diol concentration for PPD and PED. In most cases, the decrease in enzyme activity with time could be described best by bi-exponential decay, characterised by a rapid initial phase followed by a period of slower decrease in enzyme activity (Fig. 4.1). For enzyme incubated with PPD, a bi-exponential fit of the decrease in activity was only marginally better than a single exponential.

Table 4.1. Effect of diols on epoxide hydrolase stability. Enzyme was incubated in diol solutions at different concentrations at 30 °C and remaining enzyme activity was followed in time. The time required for loss of 50 % of enzyme activity is reported (t_{50}). The average first order rate constant for epoxide hydrolase inactivation in the absence of diol was 2.0 x 10^{-5} ± 8.3 x 10^{-6} min^{-1}, giving an average t_{50} of 576 h.

Diol	Diol conc. (mM)	t_{50} (h)
1-phenyl-1,2-ethanediol [a]	25	267
	50	106
	100	23
3-phenoxy-1,2-propanediol [a]	25	722
	50	48
	100	1.6
3-chloro-1,2-propanediol [a]	50	641
	100	722
glycerol [a]	100	5776
1,2-octanediol [b]	25	9
1,8-octanediol [b]	25	+ [c]
1,2-hexanediol [b]	100	93
1,2-butanediol [b]	100	+ [c]
1,2-propanediol [b]	100	+ [c]

[a] Enzyme concentration = 0.0062 mg ml^{-1}.
[b] Enzyme concentration = 0.01 mg ml^{-1}.
[c] An increase in enzyme activity was observed during incubation with diol compared to a control incubation of enzyme in buffer.

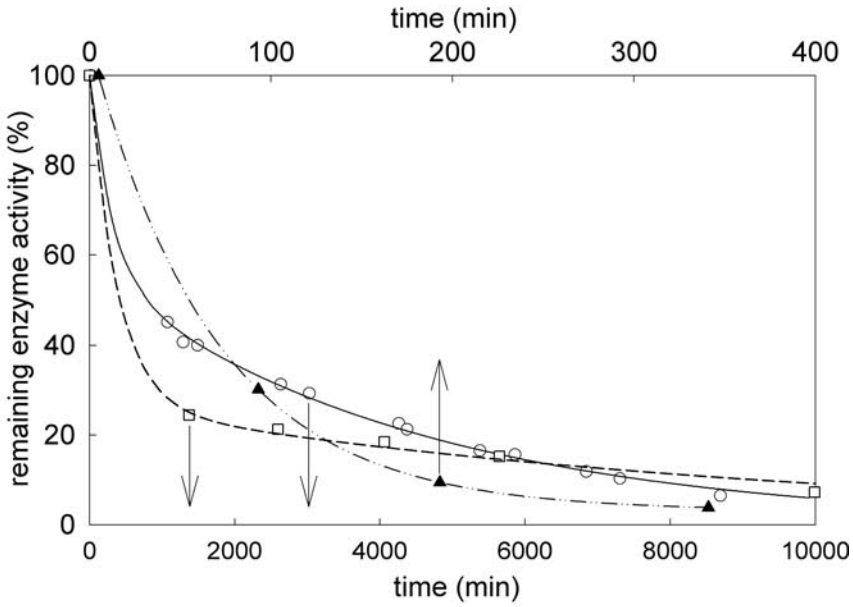


Figure 4.1. Inactivation of epoxide hydrolase by diols. Enzyme was incubated in solutions of 100 mM 1-phenyl-1,2-ethanediol (PED) (circles) or 100 mM 3-phenoxy-1,2-propanediol (PPD) (triangles) or 25 mM 1,2-octanediol (squares) and remaining enzyme activity was followed in time. Data points were fitted with bi-exponential decay curves. Arrows indicate the relevant x-axis for the data sets.

To determine whether the enzyme inactivation could be reversed, diol was removed from inactivated samples by repeated dialysis against TEM buffer. In separate experiments dialysis was carried out either at 30 ^{o}C or 4 ^{o}C to determine whether the temperature of incubation during dialysis influenced the reversibility of the inactivation.

The inactivation was partially reversible for enzyme which had been incubated in PPD when dialysis was carried out at 30 ^{o}C (Fig. 4.2). In this case, enzyme activity continued to increase slowly after diol had been removed to a maximum of 28% of the initial activity. Thus, most of the inactivation that had occurred was irreversible. The inactivation caused by PED was almost completely irreversible, regardless of the percentage decrease in activity which had occurred before diol was removed.

Partial recovery of enzyme activity was also observed after dialysis against TEM buffer at 4 ^{o}C for samples which had been inactivated in contact with PPD and 1,2-octanediol (Table 4.2). As shown in the table, the increase in activity after removal of diol was greater than what could be expected if there were only competitive inhibition by diol. Again, no recovery of activity was observed for enzyme that had been inactivated with PED.

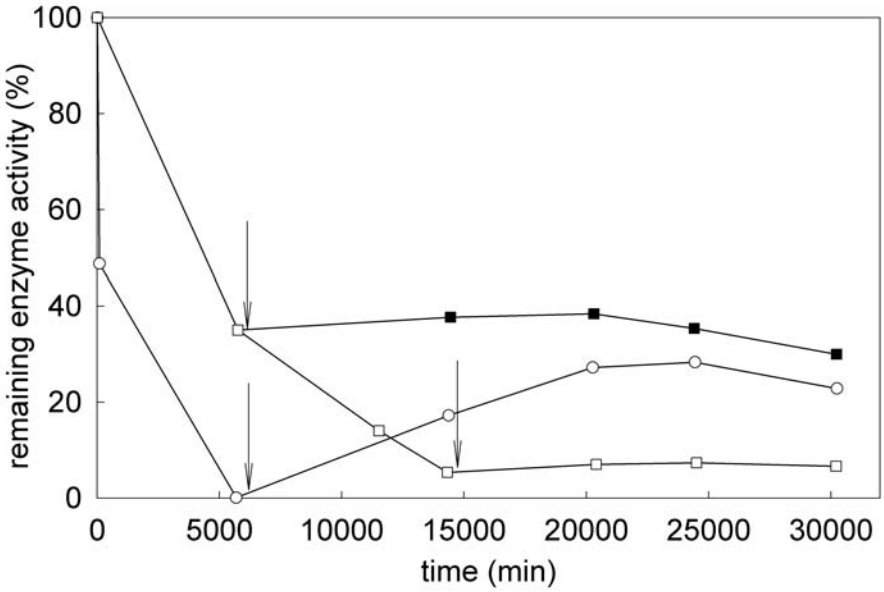


Figure 4.2. Reversibility of inactivation of epoxide hydrolase by diols. Enzyme was incubated in solutions of 100 mM 1-phenyl-1,2-ethanediol (PED) (squares) or 100 mM 3-phenoxy-1,2-propanediol (PPD) (circles) and remaining enzyme activity was followed in time. At times indicated by the arrows, the solutions were dialysed extensively against TEM buffer (pH 7.5) at 30 ^{o}C until diol concentrations were less than 0.08 μM for PPD and less than 0.02 μM for PED. After dialysis was carried out, enzyme activity was measured at regular time intervals.

Table 4.2. Recovery of enzyme activity upon removal of diol. Enzyme was inactivated by incubation in diol solutions until enzyme activity had decreased by at least 80 %. Subsequently, the diol concentration was reduced to less than 6 x 10^{-9} M by repeated dialysis against TEM buffer, pH 7.5, at 4 ^{o}C and enzyme activity was again assayed. The measured activity increase ratio was obtained by dividing the enzyme activity determined after diol removal by the activity determined after before removal.

Incubation solution	Residual enzyme activity (%)[a]		Measured activity increase ratio	Theoretical activity increase ratio [b]
	before dialysis	after dialysis		
100 mM PED	11.1	11.3	1.02	1.02
100 mM PPD	20.5	43.8	2.14	1.05
25 mM 1,2-OD	14.0	28.6	2.04	1.12

[a] Residual activity is defined as the percentage remaining activity compared to the start of incubation with diol.

[b] Expected increase in measured enzyme activity due to the removal of diol in case of competitive inhibition only, calculated from the inhibition constants (K_i, Table II) of the diols and the K_m values of the colorimetric assay substrates: *(R)*- and *(S)*-pNSO (0.008 and 0.5 mM, respectively (Rink et al., 2000)). In the assays, enzyme samples were diluted 10-fold before addition of substrate and the initial concentration of racemic pNSO was 200 μM.

Competitive inhibition

Competitive inhibition constants were determined for the diols by measuring the effect of different diol concentrations on the conversion of pNSO under first order conditions as detailed in the Materials and methods section (Table 4.3). In the assays, enzyme was added last to the reaction mixture to ensure that the time of enzyme pre-incubation with diol was kept low (< 5 min), and to minimise any effects of inactivation during the measurement of the diol binding constants (K_i). Equilibrium binding of diol to the enzyme is assumed to be complete at time zero. Inhibition constants were determined with the racemic mixtures of the diols except for 1-phenyl-1,2-ethanediol for which the constants of the *(R)* and *(S)*-enantiomers were determined individually. The observed K_i value with a racemic mixture of inhibitors will be maximally 2-fold higher than the K_i value of the most inhibiting enantiomer.

The strongest inhibitory diols were octanediols (Table 4.3). Interestingly, 1,8-octanediol had a similar inhibitory potency to 1,2-octanediol even though it is not a product of epoxide hydrolysis. Phenylacetamide, that inhibits epoxide hydrolase due to a specific interaction between the amide functionality and the active site residues (Rink et al., 2000), was a stronger inhibitor than the diols. Glycerol and 1,2-propanediol only caused inhibition at very high concentrations. The inhibitory effect of the vicinal alkanediols tested apparently increased with increasing alkyl chain length. PED also was a relatively weak inhibitor, and

inhibition by the *(R)* and *(S)*-enantiomers showed slight enantio-discrimination with K_i values of 53 and 33 mM, respectively. PPD was a stronger inhibitor than PED and CPD. The K_i value of the latter compound was close to that of 1,2-butanediol.

Table 4.3. Inhibition constants (K_i) of various compounds on wild-type *A. radiobacter* epoxide hydrolase.

Diol	Wild-type K_i (mM)	Y215F mutant K_i (mM)
1-phenyl-1,2-ethanediol (PED)	(*R*) 53	(*R*) 49
	(*S*) 33	(*S*) 31
3-phenoxy-1,2-propanediol (PPD)	7.5	6.3
3-chloro-1,2-propanediol (CPD)	34	-
glycerol	590	-
1,2-octanediol	1.5	0.8
1,8-octanediol	0.6	-
1,2-hexanediol	29	-
1,2-butanediol	50	-
1,2-propanediol	172	-
phenylacetamide [a]	0.03	0.75

[a] Data from Rink et al. (2000).

Effect of diol structure

To investigate whether enzyme inactivation was due to non-specific hydrophobic interactions between the enzyme and diols or due to a more specific interaction, the effect of alkanediols of varying alkyl chain length on enzyme stability was tested. The rate of enzyme inactivation was greater for diols with longer alkyl chains (Table 4.1). Incubation in solutions of 1,2-octanediol and 1,2-hexanediol caused a decrease of enzyme activity in a biphasic time-dependent manner (Fig. 4.1). No enzyme inactivation occurred from incubation in solutions of 1,2-propanediol or 1,2-butanediol. Instead, an increase in activity of approximately 20% with respect to the start of the incubation was observed with these diols. The cause of this observed increase in activity is not clear.

The importance of the amphiphilic character of the diol was investigated by comparing the inactivating effects of 1,2- and 1,8-octanediol. While 1,2-octanediol caused enzyme inactivation, 1,8-octanediol caused no inactivation. The latter compound even caused an increase in enzyme activity of 25% compared to the activity measured at the start of incubation. The difference in inactivating effect of the octanediol isomers could not be attributed to a difference in the binding affinity of the two diols at the enzyme active site since both octanediol isomers showed similar

potency as competitive inhibitors (Table 4.3). These results suggested that the inactivation is specific for vicinal diols and that that the hydrophobic interaction between the amphiphilic inhibitor and the enzyme plays a role in the inactivation mechanism.

Inhibitor protection from inactivation

To determine whether diol-induced inactivation resulted from diol binding at the active site, we measured the time course of enzyme inactivation in diol solutions containing a competitive inhibitor at different concentrations. Phenylacetamide was used since it is a strong competitive inhibitor of epoxide hydrolase, with a K_i value of 30 μM (Rink et al., 2000). The time course of activity decrease of epoxide hydrolase incubated with PPD (Fig. 4.3) and PED (not shown) clearly showed that addition of phenylacetamide reduced the rate of enzyme inactivation. This result provides clear evidence that diol-induced inactivation involved binding of the diol in the enzyme active site.

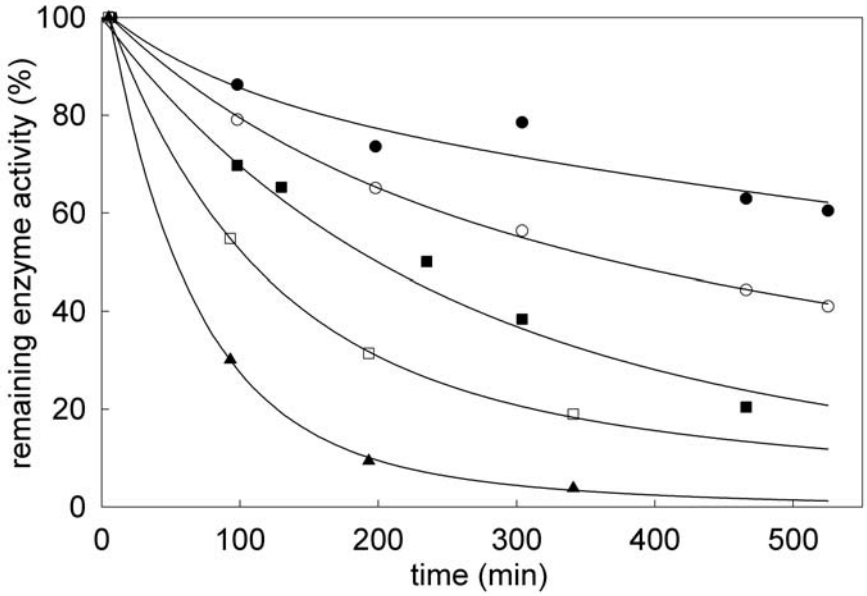


Figure 4.3. Protection of epoxide hydrolase from inactivation by a competitive inhibitor. Decrease in epoxide hydrolase activity during incubation in solutions of 100 mM 3-phenoxy-1,2-propanediol, containing the competitive inhibitor phenylacetamide at the following concentrations: (●) 1.2 mM, (○) 0.6 mM, (■) 0.3 mM, (□) 0.18 mM, (▲) 0 mM. The lines are biexponential fits of the data.

Effect of mutations in enzyme active site on diol-induced inactivation

During the formation of the alkyl-enzyme intermediate in the first step of the reaction mechanism of epoxide hydrolase, the active site residues Tyr215 and Tyr152 activate the epoxide substrate by hydrogen bonding between the epoxide oxygen and the hydroxyl groups of the tyrosines (Rink

et al., 2000) Given the active site specificity of the inactivation, as demonstrated by protection from inactivation by a competitive inhibitor, the effect of diols on the stability of the active-site mutant Y215F was investigated. The mutant enzyme was incubated in solutions of 100 mM PPD and 21 mM 1,2-octanediol and enzyme activity was measured in time. The results indicated that the mutant was resistant to inactivation by both diols. The Y215F mutant lost less than 5% activity in 120 h when incubated at 0.1 mg ml^{-1} in a 100 mM solution of PPD, while wild-type enzyme activity decreased by 90% within 25 h when incubated under the same conditions. Similarly, less than 5% decrease in activity was measured for the Y215F mutant after incubation for 19 days in 1,2-octanediol, whereas wild-type enzyme activity decreased by 55% over the same period. It is important to note that the reversible competitive inhibition of both wild-type and mutant epoxide hydrolases by PPD and 1,2-octanediol occurred approximately to the same extent (Table 4.3). This means that the mutant enzyme does not undergo inactivation even though the diols bind in the active site. These results strongly implicate the Tyr215 residue in the mechanism of inactivation of the wild-type enzyme.

Mass spectrometry of inactivated enzyme

To investigate whether enzyme inactivation by phenyl-1,2-ethanediol and other diols was accompanied by covalent modification of the enzyme, the mass of inactivated wild-type enzyme was determined by ESI-MS and compared to that of the unmodified, active enzyme. No difference in mass was found between inactivated enzyme and unmodified, active enzyme. The mass of enzyme which had been incubated in solutions of PPD, PED, 1,2-octanediol or buffer was found to be 33,901± 2 Da, which is consistent with the expected mass of 33,899 Da (wild-type protein, N-terminal methionine removed). In both control and inactivated enzyme samples, the mass spectra showed peaks with increases in mass in multiples of 97.5 ± 2.8 Da. These can be attributed to complexation with glycerol that was present in the initial enzyme storage solution. An increase in mass corresponding to covalent attachment of a diol to the enzyme was not observed for any of the diols tested, so we conclude that either no covalent bond was formed during inactivation or that it was lost either during sample preparation or during introduction of the protein into the mass spectrometer. Alternatively, the enzyme underwent a covalent modification that was not accompanied by a change of molecular mass or the irreversible inactivation caused by diols was not accompanied by a covalent modification.

Changes in enzyme structure during inactivation

To obtain greater insight into the mechanism of inactivation we investigated whether there was a correlation between the loss of catalytic activity during incubation with the diols and changes in enzyme

conformation. To this end we used circular dichroism and steady state fluorescence spectroscopy. Changes in enzyme secondary structure were monitored by taking far-UV CD spectra of samples of wild-type epoxide hydrolase that was incubated with 1,2-octanediol for different periods, while changes in enzyme activity were measured in parallel. The far-UV spectrum remained unchanged even when enzyme activity had decreased by 50% (Fig. 4.4). The intensity of the spectra also remained constant, indicating that enzyme had not aggregated and that it did not precipitate from solution during inactivation. Direct measurement of the enzyme concentration by the Bradford assay confirmed that less than 3% of the enzyme precipitated from solution during inactivation. Similarly, the far-UV CD spectra of inactivated wild-type epoxide hydrolase recovered from an incubation with 100 mM PPD, from which diol was removed by dialysis, also showed no change in the characteristic spectrum of epoxide hydrolase (not shown). From these results it is clear that the loss of enzyme activity in the presence of 1,2-octanediol is not accompanied by large conformational changes, but that it is more likely due to a localised change in enzyme structure.

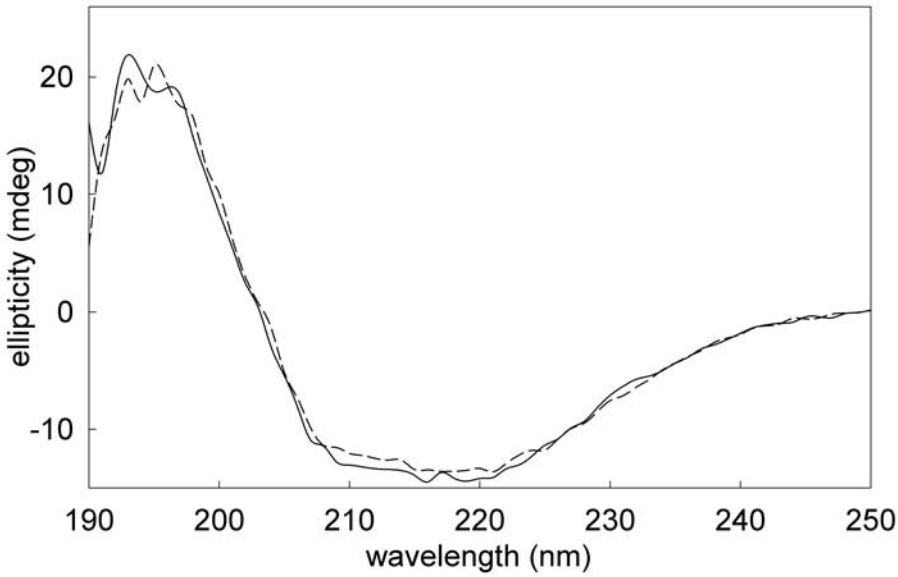


Figure 4.4. Far-UV circular dichroism spectra of epoxide hydrolase incubated in 21 mM 1,2-octanediol (dashed line) and TEM buffer (solid line). At the time of taking the spectra less than 3 % enzyme precipitation had occurred in the incubations and the enzyme activities in each incubation were (percentage relative to start of incubation): 100 % (TEM buffer) and 50 % (1,2-octanediol).

Steady-state protein fluorescence spectra are sensitive to changes in the polarity of the environment near the fluorescent tryptophans. Consequently, monitoring changes in the intrinsic protein fluorescence spectrum therefore can be useful to identify conformational changes in the enzyme. Epoxide hydrolase has eight tryptohan residues, two of which, Trp-

38 and Trp-183, are located in the active site. Just after the start of incubation, the intensity of the fluorescence emission maxima of enzyme in solutions of 100 mM PED or 25 mM 1,2-octanediol were increased by a factor of 1.3 and 1.4, respectively, compared to enzyme incubated in TEM buffer. This suggests that one or both fluorophores are in a more hydrophobic environment after diol binding. This increased fluorescence intensity was not observed for enzyme incubated in 25 mM 1,8-octanediol. During incubation in PED, the intensity of the spectra did not change in time even when 60% enzyme activity had been lost and a 15% decrease in soluble enzyme concentration had occurred. However, a slight red-shift in the maximum of the emission spectrum (337 to 343 nm) was observed, indicating that one or more of the tryptophan residues becomes exposed to water (Callis, 1997). A slight increase in fluorescence intensity with respect to control enzyme was still observed for enzyme incubated in 1,2-octanediol when activity had decreased by 50%, without any precipitation. These results indicate that inactivation is not accompanied by enzyme unfolding.

Near-UV spectra of wild-type and mutant epoxide hydrolases

Ligand-induced changes in tertiary structure can be detected using CD spectroscopy in the near-UV wavelength range (Woody and Dunker, 1996). The CD spectrum of the free enzyme was used as reference to determine if spectral changes occurred following ligand binding. The near-UV CD spectra of wild-type epoxide hydrolase and three active site mutants (Y215F, Y152F and Y215+Y152F) were compared to identify the contributions of the tyrosines in the active site. Previous analysis of far-UV CD spectra of these mutants showed that their overall structure is identical to that of the wild-type enzyme (Rink et al., 2000). Contributions of the tyrosine residues to the characteristic near-UV CD spectrum were determined by calculating difference spectra between the wild-type enzyme and each mutant. Since only partially purified enzyme preparations were used in this study (purity at least 75%) the spectra obtained should be analyzed with caution since impurities can contribute to the spectra. Nevertheless, some useful features could be identified from the difference spectra. Crystallographic data (Nardini et al., 1999) were also used in attributing spectral bands to specific residues in the interpretation of the near-UV CD spectra.

The CD spectrum of wild-type epoxide hydrolase showed a complex band of negative peaks between 250 and 295 nm and a positive band at 299 nm (Fig. 4.5). The fine structure of the spectra was similar for all enzyme forms, indicating that contributions to the spectrum from any contaminants did not excessively disturb the characteristic shape of the spectrum. The complex spectrum reflects the high proportion of aromatic residues in the enzyme: 8 tryptophans, 15 tyrosines and 14 phenylalanines. CD signals are usually influenced by interactions between aromatic groups at distances of less than 8 Å (Vuilleumier et al., 1993). Therefore from crystallographic

data, side chains of Trp38, Phe108, Tyr152, Trp183 and Tyr215 are expected to contribute greatly to the near-UV CD spectra since they form part of an aromatic cluster situated in the active site. By comparing the spectra, the negative band observed for the wild-type enzyme at 287 nm was attributed to the tyrosine residues situated in the active site. This is supported by the fact that the intensity of this distinctive band is greatly reduced for the double Y152F+Y215F mutant (Fig. 4.5). A double mutant cycle analysis was used to check for interactions between the tyrosine residues (Vuilleumier et al., 1993). It was carried out by comparing the effect on the CD spectrum of removal of the Tyr215 residue from the wild-type enzyme and from the Y152F mutant enzyme, which should be the same if there are no interactions between the two tyrosine residues. This showed that the contributions of Tyr215 and Tyr152 to the CD spectrum are not additive, suggesting that the two tyrosines interact. The negative band at 293 nm and the positive band at 299 nm are attributed to the Trp residues since phenylalanine and tyrosine residues usually do not absorb above 270 and 290 nm, respectively. The intensity of these bands is decreased in both Y215F and Y152F single mutants compared to the wild-type enzyme. This may be due to an indirect effect on the interaction of the Trp183 and Trp38 residues with their surrounding environment by the mutation of one of the two tyrosines, since these residues are all within interacting distance.

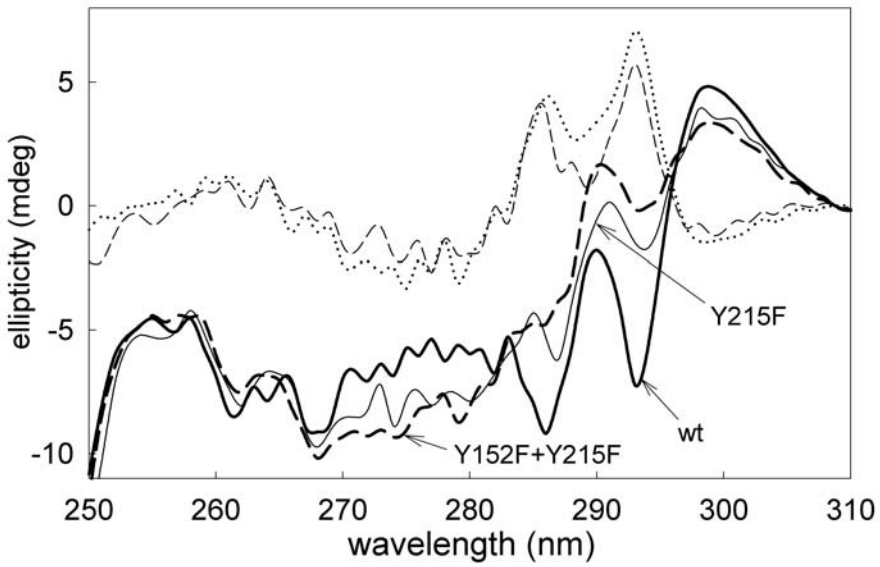


Figure 4.5. Near-UV circular dichroism spectra of epoxide hydrolase variants. Spectra of wild-type epoxide hydrolase (bold solid line), Y215F mutant epoxide hydrolase (solid line), and Y152F+Y215F mutant epoxide hydrolase (bold dashed line) in TEM buffer pH 7.5. Difference spectra for Y215F (thin dashed line) and Y152F+Y215F double mutant (dotted line) with respect to wild-type are also shown and were obtained by subtracting the spectrum of the wild-type enzyme from the spectrum of mutant enzyme.

Changes in the near-UV CD spectrum of wild-type and Y215F epoxide hydrolase incubated with 1,2-octanediol

The protection from diol-induced inactivation by the competitive inhibitor phenylacetamide showed that the inactivation of epoxide hydrolase by vicinal diols involves an interaction of the diol in the enzyme active site. Furthermore, it was found that the Y215F mutant was resistant to inactivation, suggesting that this tyrosine residue could be directly involved in the inactivation mechanism. From comparison of near-UV CD spectra of wild-type and active site mutants of epoxide hydrolase, spectral bands at 287 and 293 nm were identified as the contribution of the tyrosines and tryptophan residues in the active site respectively. Monitoring of near-UV CD spectra during inactivation was carried out to identify localized changes in structure at the active site which might occur.

Changes in the near-UV CD spectra were monitored in time for both wild-type epoxide hydrolase and the Y215F mutant incubated in a 21 mM solution of 1,2-octanediol. Octanediol was chosen for these experiments since the background signal of the incubation solution was low. At the start of incubation, the presence of saturating amounts of 1,2-octanediol (93% saturation) caused only a small alteration of the near-UV CD spectrum of wild-type enzyme. The difference spectrum between free and diol-bound enzyme showed small positive peaks at 286 and 293 nm (not shown). The spectrum of the diol-incubated enzyme changed considerably between 253 and 298 nm over the time period in which enzyme activity decreased by 55% (19 days) and soluble enzyme concentration decreased by 10% (Fig. 4.6A). In marked contrast, the spectrum of the free enzyme incubated in TEM buffer remained largely unchanged over the same time period during which enzyme activity decreased by only 9% (Fig. 4.6B). Difference spectra between diol-incubated and free enzyme at the different time points showed that significant changes in intensity occurred for bands at 287 and 294 nm, which were previously identified as the contributions of tyrosine and tryptophan residues in the active site. This suggests that the inactivation involves or influences these tyrosine and tryptophan residues. During incubation in 21 mM 1,2-octanediol the spectra of the Y215F mutant remained largely unchanged over 18 days (Fig. 4.7). As indicated above the mutant was resistant to inactivation, undergoing less than 5% inactivation during the whole time period of incubation. Together, these results suggest that the spectral changes observed for wild-type enzyme incubated in 1,2-octanediol are specific for the inactivation that takes place.

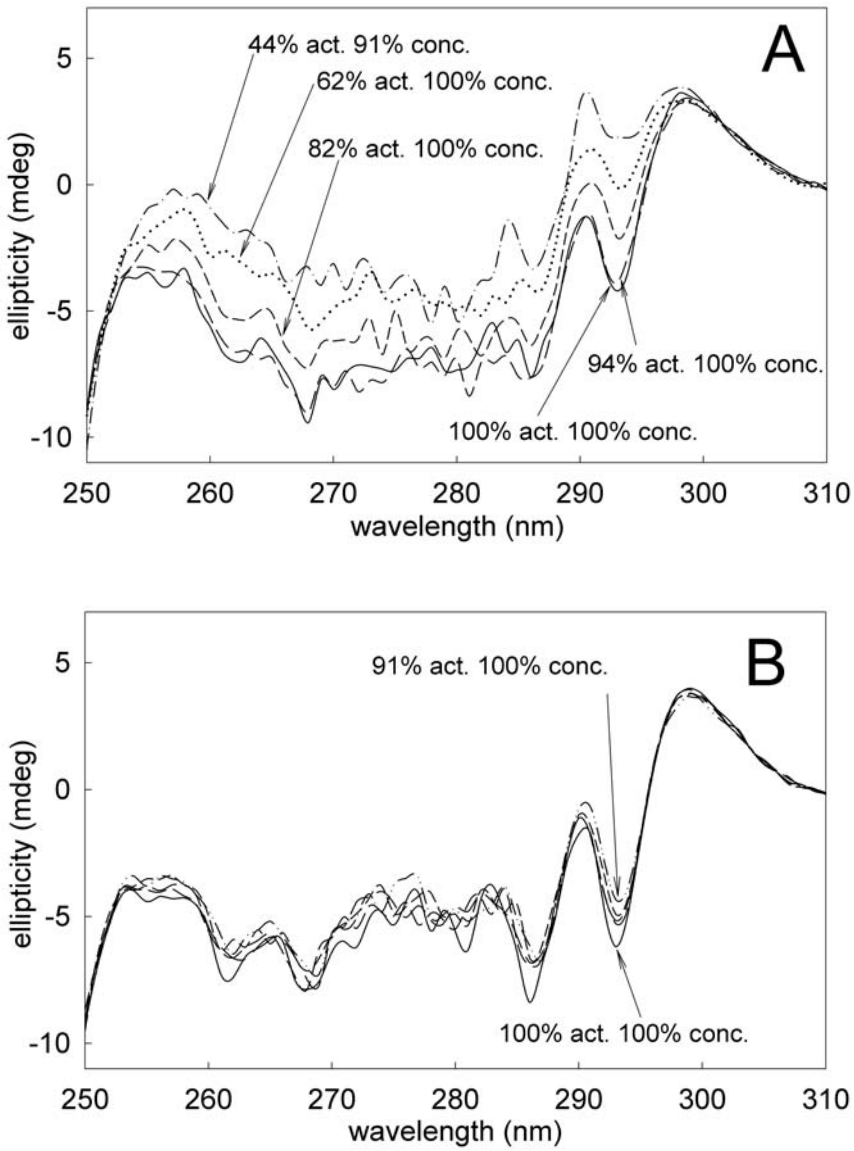


Figure 4.6. Change in near-UV spectrum of wild-type epoxide hydrolase during incubation in 21 mM 1,2-octanediol (A) or TEM buffer (B) over 19 days at 30 °C. The % remaining activity of the enzyme and the % remaining soluble enzyme concentration at the time of taking the spectra are shown.

The inactivation of wild-type enzyme by 1,2-octanediol appeared to be partly reversible (Table 4.2), so the reversibility of the change in the near-UV CD spectrum of wild-type enzyme upon incubation with 1,2-octanediol was assessed. Spectra were taken at the beginning of the incubation, after 10 and 22 days of incubation, and again after diol had been removed by repeated dialysis against TEM buffer (Fig. 4.8). The change in the spectrum was partially reversible, which is consistent with a partial reversibility of the loss of activity. This provides further evidence that the spectral change observed occurs as a direct consequence of enzyme inactivation.

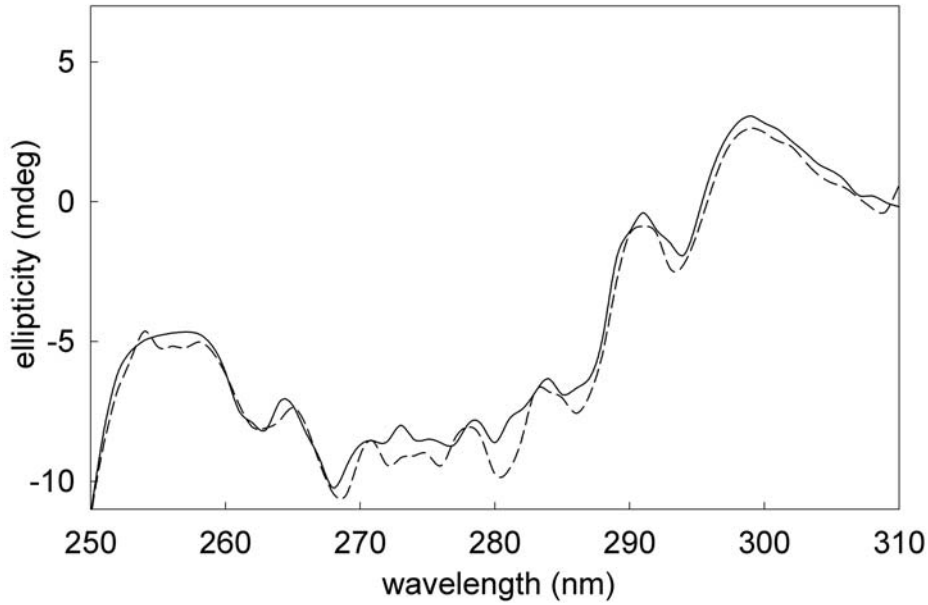


Figure 4.7. Change in near-UV CD spectrum of Y215F epoxide hydrolase mutant during incubation in 21 mM 1,2-octanediol (full line at time = 0, dashed line after 18 days)

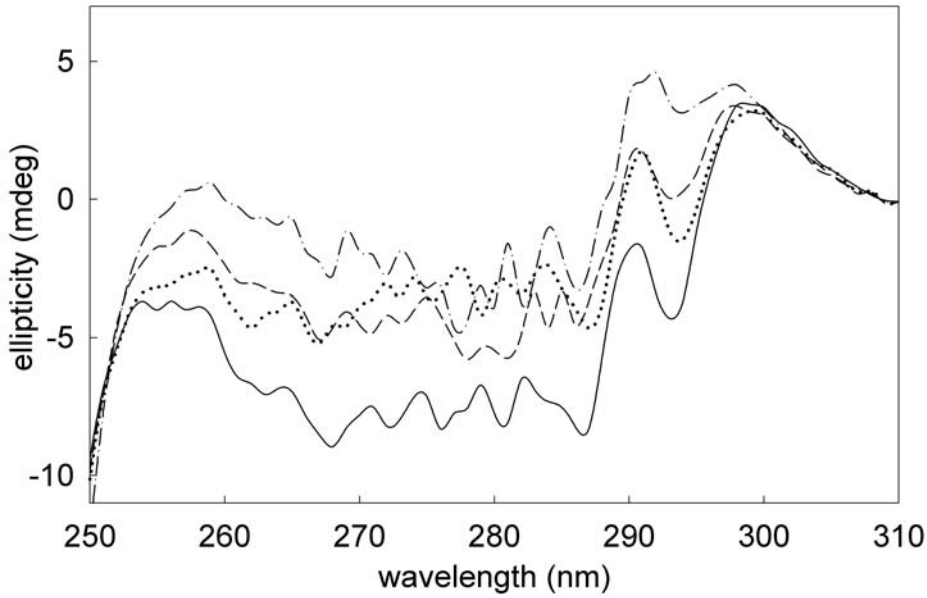


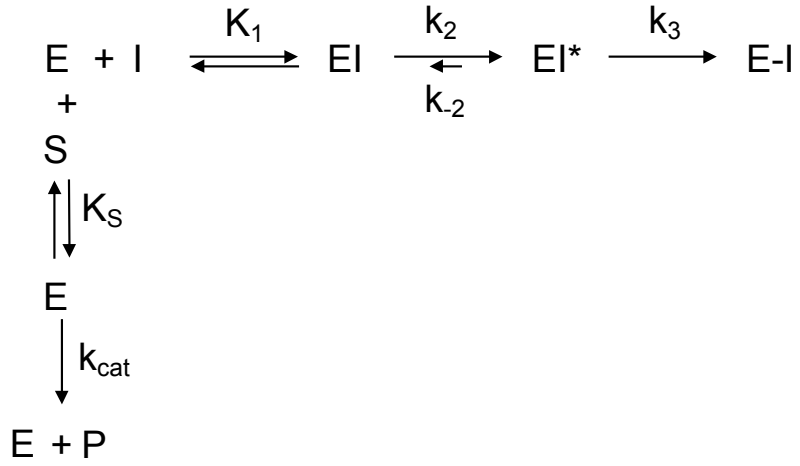
Figure 4.8. Reversibility of changes in near-UV spectra of wild-type epoxide hydrolase incubated in 21 mM 1,2-octanediol. Full line spectrum taken at t=0; dashed line spectrum taken after 10 days; dash-dot-dash line spectrum taken after 22 days incubation. Dotted line shows spectrum taken after removal of diol by repeated dialysis against TEM buffer.

Discussion

Putative kinetic mechanism of inactivation

Based on the observed biphasic time courses of inactivation during incubation of enzyme in diol solutions, the inactivation of epoxide hydrolase by vicinal diols appears to occur by a three step mechanism (Scheme 4.1). In the first step, rapid and reversible binding of diol at the enzyme active site occurs to form a complex EI (step 1). During the enzyme activity assay, which was measured with a colorimetric substrate, enzyme in the EI form can rapidly dissociate and take part in substrate turnover as free enzyme (E). The positive correlation between inactivating efficiency and inhibitory potency of the diols (Table 4.3) can be partly explained by the fact that for an equal diol concentration the enzyme is saturated to a greater extent by diols that are stronger competitive inhibitors.

Scheme 4.1. Proposed kinetic mechanism of inactivation of epoxide hydrolase by vicinal diols (I). During activity assays the diol competes with the epoxide substrate (S) for binding at the enzyme active site. Inactivation proceeds by rapid reversible binding of the inhibitor, followed by a two-step process leading to irreversibly inactivated enzyme (E-I).



Hydrophobic interactions could play a significant role in determining initial and reversible binding of diols in the active site. Docking studies of the strong inhibitor phenylacetamide in the active site of this epoxide hydrolase positioned the aromatic segment of the molecule in a highly hydrophobic pocket (Nardini et al., 2001). Comparison of the inhibition constants of phenylacetamide and acetamide for wild-type epoxide hydrolase (0.03 and 37 mM respectively) indicated that the presence of a phenyl group contributed significantly to the binding efficiency (Rink et al., 2000). Such hydrophobic interactions probably also help to stabilise diol binding in the active site, explaining the greater diol inhibitory potency observed for alkanediols with a larger hydrophobic moiety (Table 4.3). For relatively

weak inhibitors such as PED and PPD, the near equality of the inhibition constants obtained for the wild-type and Y215F mutant enzymes (Table 4.3) could then be partly explained by the fact that this non-specific driving force for binding is unchanged between the enzyme forms. A further stabilisation of diol binding in the active site may be afforded by polar interaction with the tyrosines in the active site. For example for PPD, interaction between the tyrosines and the non-hydroxyl oxygen atom of PPD could explain the slightly better binding of PPD compared to PED.

The slow bi-exponential nature of the enzyme activity decay curves implies that step 1 is followed by at least two slow sequential steps. Partial recovery of enzyme activity was possible upon removal of diol by repeated dialysis on a time-scale of days. These results suggest that step 2 is only slowly reversible, such that enzyme bound in the EI* complex does not participate in substrate turnover in the activity assays since the equilibrium between EI and EI* is slow relative to the assay period. The reversibility varied for the different diols. For example, the remaining activity of an enzyme sample which had been inactivated with PPD slowly increased from 3% before dialysis, to a maximum of 28% after 10 days after dialysis. This indicates that when the remaining enzyme activity had decreased to 3%, a proportion of enzyme was in the EI* form which then reversed to give active enzyme after removal of diol. Partial reversibility was also observed with 1,2-octanediol. In contrast, activity of enzyme that had been incubated in PED could not be recovered even after extensive dialysis, suggesting that for this diol step 2 is nearly irreversible (k_{-2}).

To account for the final irreversibility of loss of activity, the complex E-I (enzyme-diol) must be formed irreversibly (step 3) so that enzyme in this form is incapable of turning over substrate in the activity assays and no activity is recovered during prolonged incubation after dialysis. Step 3 also appears to be very slow. Several hypotheses can be proposed regarding the precise nature of the slow irreversible step in the inactivation mechanism.

One hypothesis is that the diol stimulates an irreversible conformational change which appears to occur locally in the enzyme active site and is only partially reversible. ESI-MS data would suggest that this occurs without the formation of a covalent adduct that is stable. The precise nature of such a conformational change is hard to envisage, however. X-ray structural data of the wild-type epoxide hydrolase have shown that the enzyme can undergo conformational changes in the active site (Nardini et al., 1999 and 2001). It was shown that in the native structure a conformational change has placed the side chain of Gln134 into the active site, occupying the site for substrate binding. In this structure, the position of the Gln side chain was stabilized by hydrogen bonding with Tyr152, Tyr215 and Asp107 (Rink et al., 2000). This conformational change was ascribed to crystal contacts and the affinity of the active site for amide compounds, and it is possible that this same change in active site

architecture occurs during inactivation. However, such a conformational rearrangement is not expected to be irreversible, suggesting that there maybe a further reaction in the active site. How the presence of diol would stimulate this structural rearrangement is also unclear, but the increase in enzyme steady state fluorescence intensity in the presence of diol may indicate that conformational changes yielding a more hydrophobic environment or the exclusion of quencher groups in the vicinity of tryptophan residues occurs immediately upon diol addition. A similar increase in fluorescence intensity and red-shift of 5 nm in fluorescence maximum was observed during conversion of (*R*)-styrene oxide (Rink and Janssen, 1998a). This was attributed to a unimolecular isomerization step occurring after the formation of the covalent intermediate, although the nature of this step was not fully characterized. Thus, the current knowledge of the conformational change involving Gln134 does not provide a good basis for explaining the observed irreversible diol-induced inactivation described in this chapter.

Alternatively, the observed inactivation may be related to a chemical change that occurs as a consequence of diol binding in the active site. The observed irreversibility lends support to this possibility, although no covalent adduct was identified by mass spectroscopy data. One hypothesis for the mechanism of the covalent binding is the formation of an ester bond between the carboxylic side chain of Asp107, one of the catalytic triad residues, and the diol (Figure 4.9, step 1), which is the reverse of the hydrolysis step in the conversion of epoxide substrate (Rink and Janssen, 1998a). Since this would not normally be irreversible, an 'abnormal' ester would have to be formed with the secondary hydroxyl, which may be only slowly hydrolysed (Figure 4.9, step 3). Alternatively, a structural rearrangement of the bound diol would have to take place (Figure 4.8, step 2) by attack of the free hydroxyl group of the bound diol on the carbonyl function of Asp 107, which would be irreversible or only very slowly reversible. The rearrangement may be facilitated by the presence of Tyr215 by abstracting a proton from the free hydroxyl, or by donating a proton to the leaving group oxygen. Furthermore, the presence of diol at high concentrations may stabilise the Michaelis complex (1) and the ester intermediate (2) in the active site sufficiently for the successive modifications to take place. In their study of the reaction mechanism of microsomal epoxide hydrolase, Tzeng et al. (1998) proposed a similar acyl migration in the ester intermediate as a competing reversible reaction to the hydrolytic half reaction in the mechanism. A similar chemical step was proposed as one of the possibilities that constitutes the extra unimolecular isomerisation step identified in the four-step mechanism for the conversion of (*R*)-styrene oxide by this epoxide hydrolase (Rink and Janssen, 1998a). The kinetics of such a modification could be complex since the different diol enantiomers could have different capabilities for forming the covalent

adduct and subsequent rearrangement. The rearrangement could proceed at a higher rate depending on the nature of the substituent groups of the different diols or even the enzyme environment, resulting in the different inactivation rates observed. The formed ester bond could be rapidly hydrolyzed in the acidic conditions used for sample preparation for the determination of enzyme molecular mass by ESI-MS, which may explain why no change in enzyme mass was detected by this method.

An alternative hypothesis for an irreversible step could involve activation of Tyr215 for nucleophilic attack on the carbonyl carbon of the ester (Figure 4.8 B) forming a complex which would not result in an increase in enzyme molecular mass. However, this possibility may be unlikely due to the distance between Tyr215 and Asp107 (6 Å) (PDB accession code 1EHY).

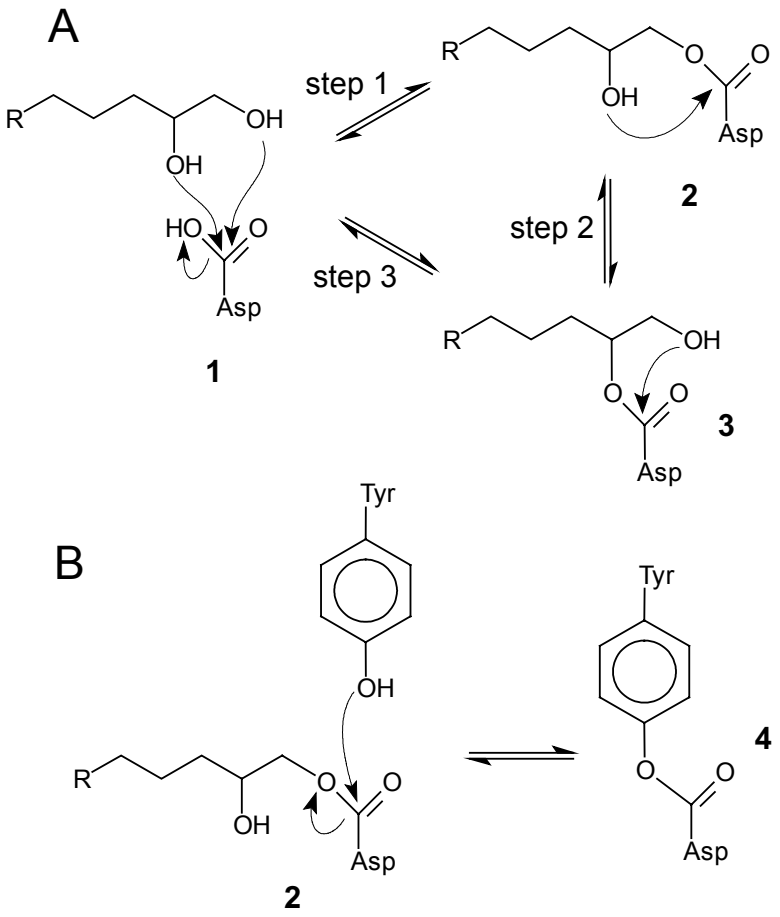


Figure 4.9. Schematic representation of the putative mechanisms for the irreversible step during inactivation of epoxide hydrolase by vicinal diols. Panel A shows the possible formation of a covalent ester intermediate (step 1) followed by rearrangement of the diol into a form which is not readily hydrolyzed (step 2); or the direct formation of an 'abnormal' ester via the secondary hydroxyl (step 3). Panel B shows the alternative possibility of direct nucleophilic attack by the Tyr215 oxygen on the carbonyl carbon of the ester. Instead of a tyrosine, another nucleophilic group could possibly attack the covalent intermediate. This mechanism would account for covalent modification with only a small change of molecular mass.

Conclusions

Epoxide hydrolase undergoes time-dependent irreversible loss of activity upon incubation with vicinal diols. Inactivation did not cause enzyme unfolding but was accompanied by a localised change in enzyme structure at the active site. The active site tyrosine mutant (Y215F) was resistant to inactivation by diols indicating that the presence of the two tyrosines in the active site is necessary for inactivation. The findings in this investigation have implications for the optimisation of large-scale kinetic resolution of racemic epoxides. To maintain high enzyme activities during conversion the concentration of diol should be kept low or substrates should be chosen of which corresponding diols are poor enzyme inhibitors. Importantly we have shown that the Y215F mutant is stable when incubated in diol solutions at high concentrations. This is a good example of the use of site directed mutagenesis to increase biocatalyst stability with respect to chemical inactivation. Since this mutant enzyme also showed an increased enantioselectivity compared to the wild-type enzyme (Rink et al., 1998b, Rink et al., 1999) it should be the preferred biocatalyst for application in large-scale epoxide kinetic resolutions.

Chapter 5

Stable and enantioselective cross-linked epoxide hydrolase crystals

Abstract

In this chapter the formation and application of cross-linked crystals of epoxide hydrolase to the kinetic resolution of racemic epoxides is described. A highly enantioselective epoxide hydrolase active site mutant (Y215F) was crystallised by batch crystallisation. Cross-linking the crystals with glutaraldehyde permitted the formation of a stable biocatalyst which was resistant to dissolution in aqueous medium. The cross-linked epoxide hydrolase crystals (CLEhCs) could be used to carry out enantioselective kinetic resolution of epoxides, resulting in epoxides of high enantiomeric purity. The enantioselectivity of the cross-linked crystals for styrene oxide and *p*-nitrostyrene oxide was lower than that of the soluble form of the enzyme. The decreased enantioselectivity was at least partially caused by intra-CLEhC mass transfer limitations. Although the cross-linked epoxide hydrolase crystals had a lower specific activity than the soluble enzyme, CLEhCs could be reused several times for batch kinetic resolutions. Compared to the soluble enzyme, the cross-linked crystals showed higher thermostability at 60 ^{o}C, increased stability when incubated with 50% DSMO and no inhibition by diols. The results indicate that CLECs of this epoxide hydrolase mutant are promising for carrying out large-scale kinetic resolutions.

Introduction

Cross-linked enzyme crystals (CLECs) are formed by cross-linking enzyme microcrystals using a bifunctional reagent. The intermolecular cross-links maintain the crystal structure in solutions of composition different to the crystallization liquor (Margolin, 1996) and the enzyme is stabilised by numerous electrostatic and hydrophobic interactions which arise due to the very high enzyme concentration in the crystals (Tischer et al., 1999). CLECs have been shown to possess increased stability in the presence of organic cosolvents and at high temperatures compared to the respective enzymes in solution (Margolin, 1996; Noritomi et al, 1998) and have therefore become attractive as a form of immobilised biocatalyst for use under reaction conditions which may cause loss of biocatalyst activity. Compared to soluble enzymes, CLECs have the additional advantage of being easy to recover and recycle. CLECs also generally have a higher specific activity by weight than immobilised enzymes where the carrier makes up a large proportion of the catalyst.

Margolin and Navia (2001) have given a comprehensive review of CLEC technology applied to biocatalysis. CLEC technology has been commercialised by Altus Biologics Inc. (Cambridge, MA) for application in biotransformations and protein drug delivery. CLECs of lipases, proteases and esterases are some of the enzymes currently sold for biocatalytic application. Recently, the potential for using CLECs as a selective crystalline chromatographic material for chiral separation has been demonstrated (Margolin, 1999; Pastinen et al., 2000). Cross-linked protein crystals can also be used for the controlled release (St. Clair et al., 1999) of therapeutic proteins in medical applications, since crystal dissolution can be carefully controlled by the method of cross-linking and the nature of the incubation environment.

The remarkable reported stability of CLECs prompted us to evaluate the possibility of forming cross-linked crystals of epoxide hydrolase for application in the kinetic resolution of racemic epoxides. The enantioselective hydrolysis of racemic epoxides by an epoxide hydrolase from *Agrobacterium radiobacter* AD1 can be carried out at high substrate concentrations in a biphasic liquid system (Baldascini et al., 2001). It has been shown, however, that in such a system epoxide hydrolase inactivation can occur by direct contact of the enzyme with the organic liquid (Baldascini and Janssen, 2004) and by accumulation of the formed diol product in the aqueous phase at high concentrations (Chapter 4). Our aim was to create cross-linked crystals of epoxide hydrolase and determine their activity and enantioselectivity. Furthermore, the possibility for their reuse in successive batch conversions was also investigated.

Disadvantages recognised in the use of CLECs include the lower specific activity compared to free enzyme. This is sometimes attributed to

reduced mass transfer rates of substrates and products in the crystal channels (Margolin, 1996) or to alteration in enzyme conformation during cross-linking (Costes et al., 2001). In immobilised enzyme systems, mass transfer limitations can reduce the observed enzyme selectivity (Barros et al., 2000). Previously, we have shown that mass transfer limitations reduce the effective enantioselectivity of kinetic resolutions if the enzyme does not have absolute enantioselectivity. The resolution of racemic styrene oxide by wild-type epoxide hydrolase is an example of this, where the preferred (*S*) substrate enantiomer inhibits the conversion of the (*R*)-enantiomer which is converted last (Baldascini et al., 2001). Mass transfer limitations would result in a reduction in yield of the remaining epoxide substrate enantiomer, which is clearly undesirable. To reduce the impact of potential mass transfer limitations in this work, we used an active site mutant of this epoxide hydrolase (Tyr215Phe) which converts several substituted styrene epoxides with a much higher intrinsic enantioselectivity than the wild-type enzyme (Rink et al., 1999). For some substrates e.g. *p*-chlorostyrene oxide, the rate of conversion of the second enantiomer is reduced virtually to zero so that mass transfer limitations would have little effect on effective enantioselectivity of the reaction. The reduced specific activity observed with the mutant enzyme would also tend to make the system kinetically limited rather than mass transfer limited, even at the high enzyme concentrations encountered in CLECs. In addition, it has been shown that the Y215F epoxide hydrolase mutant is resistant to inactivation by vicinal diols at high concentrations (*Chapter 4*) which makes it a preferable for use in large-scale applications.

Batch crystallisation is the most suitable method to obtain large amounts of protein crystals (Lee et al., 2000). Screening for the best conditions for crystallisation is carried out in the same manner as in the production of crystals for X-ray crystallographic studies. Conditions such as temperature, pH, precipitant type and concentration, protein concentration and purity and salt concentration are tested using the hanging drop crystallisation method. These conditions are then used as first guesses for batch crystallisation trials, and can be further optimised by experimental design. In batch crystallisation, seeding and rate of mixing are additional parameters that affect the crystallisation process (Margolin 1996). Crystallisation should be optimised to maximise crystallization yield and most importantly the crystallisation should be reproducible and amenable to scale-up. Crystal properties which impact the functionality of the CLECs are size and uniformity of size. In general, large crystals of thickness greater than 5 μm can potentially suffer from mass transfer limitations (Govardhan, 1999). The length of the crystals on the other hand has to be large enough (≥ 150 μm) to ensure good recovery of CLECs by filtration to avoid large losses of CLEC material during recycling (Collins et al., 1998).

Cross-linking of enzyme crystals is often carried out using the homobifunctional reagent glutaraldehyde which reacts with the ε-amino groups of lysine (De Santis and Jones, 1999). The inter- and intramolecular cross-linking helps to resist unfolding at high temperatures and in unfavourable environments. Glutaraldehyde can form oligomers of different length and therefore can form intramolecular cross-links spanning existing distances between lysine residues, reducing possible distortion of the protein conformation (Perischetti et al., 1995). The reduction in specific activity of CLECs, which is frequently observed compared to enzyme in solution, is often explained by a decrease in conformational flexibility of the enzyme in the crystalline state. In addition, the catalytic mechanism may be altered in the formation of CLECs if an essential residue is modified during cross-linking (Quiocho and Richards, 1966). The degree of cross-linking is determined by the cross-linking reagent concentration, cross-linking time, temperature and pH. A trade-off between stability of the crystals and activity has been reported as a function of the degree of cross-linking and the optimal degree of cross-linking seems to depend on the particular protein and crystal form (Margolin, 1996). For large-scale applications, any potential reduction in catalytic activity of CLECs can be offset by their increased stability and reusability.

In this chapter, the identification of suitable batch crystallisation conditions for the Y215F epoxide hydrolase mutant is described. Batch crystallisation was carried out at an intermediate scale of 1 ml, which is enough for a preliminary investigation into optimising cross-linking conditions and for assessing the catalytic activity, enantioselectivity, stability and reusability of the cross-linked enzyme crystals. Cross-linking of the crystals was carried out using glutaraldehyde with the objective of maximising retention of catalytic activity. The enantioselectivity of the cross-linked epoxide hydrolase crystals (CLEhCs) was determined by performing kinetic resolutions of two racemic epoxides (*p*-nitrostyrene oxide and styrene oxide) and conversions of the epoxides in their enantiopure forms. In addition, thermostability of the CLEhCs was compared to that of the soluble form of the enzyme.

Material and Methods

Enzyme preparation and chemicals

The Y215F epoxide hydrolase was produced using a recombinant *E. coli* strain and purified by a two-step chromatography procedure as described by Rink et al. (1997). An initial purification step was carried out using a DE52 anion exchange column. The second purification step involved the use of a hydroxylapatite column. The purified enzyme was dialysed

against TEMA (25 mM Tris-SO_4, 1 mM EDTA, 1 mM mercaptoethanol, 0.02% sodium azide) buffer at pH 7.5.

Racemic styrene oxide (98%), (*R*)-styrene oxide (93% e.e., determined by GC) and (*S*)-styrene oxide (99% e.e., determined by GC) were obtained from Sigma-Aldrich. *p*-Nitrostyrene oxide (pNSO) was synthesised as described elsewhere, and (*R*)- (99% e.e., determined by HPLC) and (*S*)-pNSO (99% e.e., determined by HPLC) were obtained by separation of the enantiomers by preparative chiral HPLC (Lutje Spelberg et al., 2001). Glutaraldehyde, PEG 8000, NaCl, and imidazole were obtained from Sigma.

Analytical procedures

Samples for the determination of styrene oxide concentrations were prepared by withdrawing 50 µl of the reaction mixture and extracting with 300 µl of diethylether containing mesitylene as internal standard. Chiral analysis was carried out with a Helwett-Packard 5890 gas chromatograph with an FID-detector, using a Chirasil Dex CB (Chrompack) column. Samples for the determination of pNSO concentrations were prepared by withdrawing 50 µl of the reaction mixture and extracting with 250 µl of n-octane containing mesitylene as internal standard. The samples were analysed by chiral HPLC on a Chiralpak AS (Daicel) column with UV detection at 254 nm. Isocratic elution with hexane:2-propanol (ratio 9:1) was carried out at a flow rate of 1.3 ml min^{-1} (retention time (*R*) = 10.5 min and retention time (*S*) = 15.7 min).

Batch crystallisation

Enzyme was crystallised by using a batch crystallisation method. The crystallisation conditions which had been used successfully to crystallise the Y215F epoxide hydrolase mutant by the hanging drop method were used for the initial screening. Optimal crystallisation conditions were screened by varying the concentration of enzyme and polyethyleneglycol (PEG 8000) in the crystallisation mixture. Enzyme concentration was varied between 2 mg ml^{-1} and 5.3 mg ml^{-1} and the PEG 8000 concentration was varied between 16 and 26% w/v. Imidazole and NaCl concentrations were fixed at 100 mM and 200 mM, respectively, and the crystallisation was carried out at pH 8.4 and a temperature of 22 ^{o}C. The screening was carried out using an Oryx 6 crystallisation robot (Douglas Instruments) using crystallisation liquor droplets of 2.2 µl total volume.

At the 1 ml scale, crystallisation was carried out in solution containing 100 mM Imidazole (pH 8.4), 200 mM NaCl and 24% w/v PEG, with an enzyme concentration of 4 mg ml^{-1}. All the crystallisation liquor components were mixed thoroughly together before adding enzyme to the desired concentration. Crystals were grown at 22 ^{o}C.

Cross-linking of crystals

Cross-linking of crystals was carried out with glutaraldehyde. Crystals were transferred (using a micropipette) to 200 µl liquid of the same composition as the crystallisation liquor which also contained glutaraldehyde at a concentration of 5% v/v (25% stock solution). Cross-linking was carried out in wells of a 96-well plate which was UV grade allowing the visual inspection of the CLEhCs through a microscope using polarised light. The crystals were allowed to cross-link for 0.5 h, 1 h or 24 h. The CLEhCs were washed five times with 400 µl TEM buffer (50 mM Tris-SO_4, 1 mM EDTA, 1 mM mercaptoethanol, pH 7.5) by repeatedly adding fresh buffer to the plate well, mixing by repeated injections with the Gilson pipette, allowing the crystals to settle and removing the wash liquid with a Gilson pipette. The CLEhCs were stored at room temperature until required.

Activity assays

Activity assays were carried out by following the hydrolysis of the colorimetric substrate pNSO at 310 nm. The extinction coefficients for the epoxide and the corresponding diol are ε_{310} = 4289 $M^{-1}cm^{-1}$ and ε_{310} = 3304 $M^{-1}cm^{-1}$, respectively (Rink et al., 1999). Assays were carried out in 96-well plates to enable several reactions to be carried out simultaneously. The small well volume made it possible to measure reasonable reaction rates with very small amounts of CLEhCs. The plates were read using a PowerWaveX plate reader (Bio-tek Instruments, Vermont, USA) with periodic shaking during readings. In each well, the reaction mixture consisted of 200 µl TEM buffer, pH 7.5 and a similar amount of CLEhCs. Assays were started by adding of 1µl of a 30 mM stock solution of pNSO dissolved in acetonitrile. Activity was determined by calculating initial reaction rates for no more than 20% substrate conversion.

CLEhC reusability was tested by repeating assays, performed as described above, using the same batch of CLEhCs in the reaction mixture. Between successive assays the reaction buffer was removed and the CLEhCs were washed five times with 400 µl of fresh buffer as described above.

Enantioselectivity

Kinetic resolutions were performed with racemic pNSO and racemic styrene oxide at initial substrate concentrations of 1 mM. Enzymatic conversion of the enantiomers of each epoxide was also tested separately at initial concentrations of 0.5 mM. For each epoxide a dedicated batch of CLEhCs was used for the kinetic resolution reaction followed by the single enantiomer conversions. The batches were washed thoroughly with TEM buffer between reactions. The reaction mixtures consisted of 1.5 ml TEM buffer (pH 7.5) containing a similar amount of CLEhCs. The reactions were carried out at room temperature (22 ^{o}C) and were started by adding pNSO from a stock solution in acetonitrile (final acetonitrile conc. < 1% v/v), or

styrene oxide from a concentrated aqueous solution, to the desired initial substrate concentration. All incubation vials were stoppered and shaken using a mechanical vortex shaker at a fixed rotational speed which maintained suspension of the crystals in solution. Substrate conversion was followed by periodically taking 50 μl aliquots of the reaction mixture and the samples were analysed as described above. To avoid withdrawing CLEhCs while sampling, the mixing was stopped and crystals allowed to settle during 1 min before withdrawing liquid.

CLEhC stability

Stability of the CLEhC preparation was tested by performing activity assays in the 96-well plates using the colorimetric substrate pNSO as described above. Stability was determined by measuring the change in activity of a CLEhC batch before and after incubation under a given set of conditions. Activity was determined by calculating initial reaction rates for no more than 20% substrate conversion. In this way, CLEhC themostability was tested by incubating the CLEhCs at 60 ^{o}C and measuring activity after 1.5 h and then 5 h. After the first activity assay the CLEhCs were washed with TEM buffer before re-incubation at 60 ^{o}C. Similarly CLEhC stability was tested during incubation with 50% DMSO and during incubation in solution containing 100 mM 3-phenoxy-1,2-propanediol.

Soluble epoxide hydrolase thermostability

Thermostability of soluble Y215F was measured by incubating a 0.2 mg ml^{-1} solution of the mutant in TEM buffer at 50 ^{o}C or 60 ^{o}C and following the change in enzyme activity with time. The thermostability of the wild-type epoxide hydrolase was similarly assessed at 50 and 60 ^{o}C by incubation in TEM buffer at the same concentration as the mutant enzyme. Periodically, enzyme activity was assayed by following the conversion of the colorimetric substrate pNSO as described elsewhere (Baldascini et al., 2001). Samples from the enzyme incubation were diluted with TEM buffer so that the assay mixture reached room temperature before the addition of pNSO.

Product inhibition

The inhibitory effect of diols on CLEhC activity was determined by measuring the change in CLEhC activity between incubation in TEM buffer and incubation in diol solutions at different concentrations. The activity of a CLEhC batch was determined by the colorimetric assay as described above. After the assay, the CLEhC batch was washed with TEM buffer 5 times and CLEhCs were resuspended in a diol solution, whereupon the activity assay was repeated in the same manner. The change in activity was expressed as a percentage change from the activity in TEM buffer.

Results and Discussion

Batch crystallisation

The remarkable stability commonly reported for CLECs prompted us to evaluate the use of cross-linked crystals of epoxide hydrolase for the kinetic resolution of racemic epoxides. Batch crystallisation is a crystallisation method which is generally amenable to scale up (Lee et al., 2000) and was therefore used to obtain crystals of the epoxide hydrolase. Batch crystallisation conditions were screened by varying enzyme and PEG 8000 concentrations simultaneously, with fixed imidazole and NaCl concentrations at pH 8.4. The enzyme crystallised over a broad range of concentrations for the parameters varied (Fig. 5.1). The time required for crystals to appear was dependent on both enzyme and precipitant concentrations, with the first crystals appearing within 24 h. Conditions which generated the most uniform crystals in a reasonable amount of time were chosen to scale up the crystallisation to 1 ml. Batch crystallisation at the 1 ml scale was carried out at 4 mg ml^{-1} enzyme concentration and 24% w/v PEG. At this scale, crystals appeared within 8 weeks and were small plates which were left to grow to a maximum size of 400 μm in length, 200 μm in width and approximately 40 μm thick.

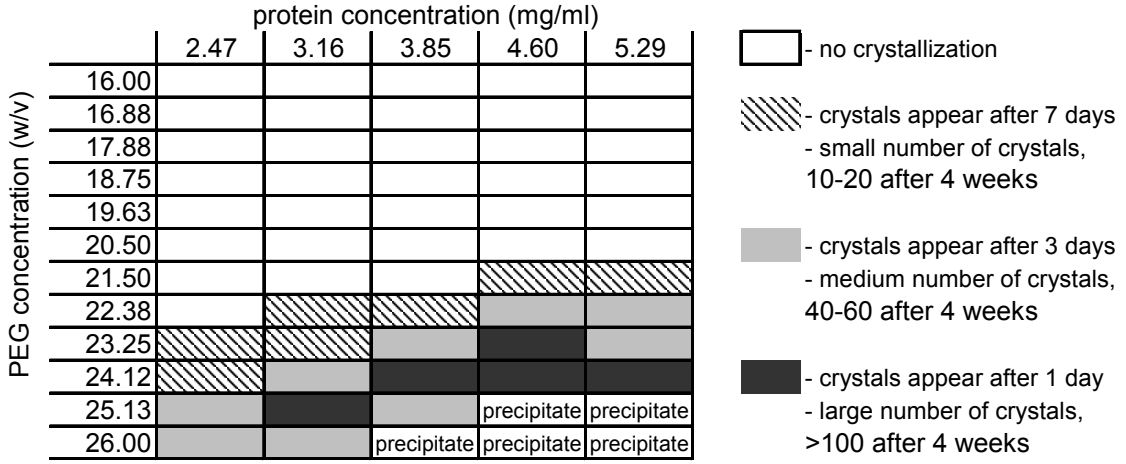


Figure 5.1. Screening of batch crystallisation conditions for Tyr215Phe mutant epoxide hydrolase. PEG 8000 and protein concentrations were varied as shown, with fixed imidazole (100 mM, pH 8) and NaCl (200 mM) concentrations. Screening was carried out using an Oryx 6 crystallisation robot (Douglas Instruments) with crystallisation liquor droplets of 2.2 μl total volume.

The crystallisation at the 1 ml scale was repeated with a second batch of purified epoxide hydrolase under the same conditions used for the first 1 ml crystallisation. The crystallisation in the second batch gave a relatively low yield of needle shaped crystals, and the formation of a precipitate and a skin layer were noted. The presence of contaminants in a protein solution

can have negative consequences on the ability to crystallise a protein. It is possible that a small variation in the epoxide hydrolase batch purity caused the poor repeatability of the crystallisation step.

Cross-linking

Crystal cross-linking was carried out with 5% v/v glutaraldehyde. Cross-linking times of varying duration were tested to determine the effect of degree of cross-linking on CLEhC activity. Activity assays (see below) were carried out in a convenient 96-well plate format. Approximately the same number of crystals was contained in each well as determined visually by microscope observation. The substrate concentration profiles recorded showed some scatter in the recorded absorbance (Fig. 5.2) which was attributed to interference by CLEhCs in the light path. The degree of scatter in the data was variable from well to well. Despite this scatter it was observed that CLEhC activity decreased with increasing cross-linking time (Fig. 5.2). When crystals were cross-linked for 24 h, conversion of pNSO proceeded at a rate equal to spontaneous pNSO hydrolysis, indicating that the CLEhCs were completely inactive. Crystals which were cross-linked for the shortest time (0.5 h) showed the highest activity. CLEhCs used in all further experiments were cross-linked for 0.5 h.

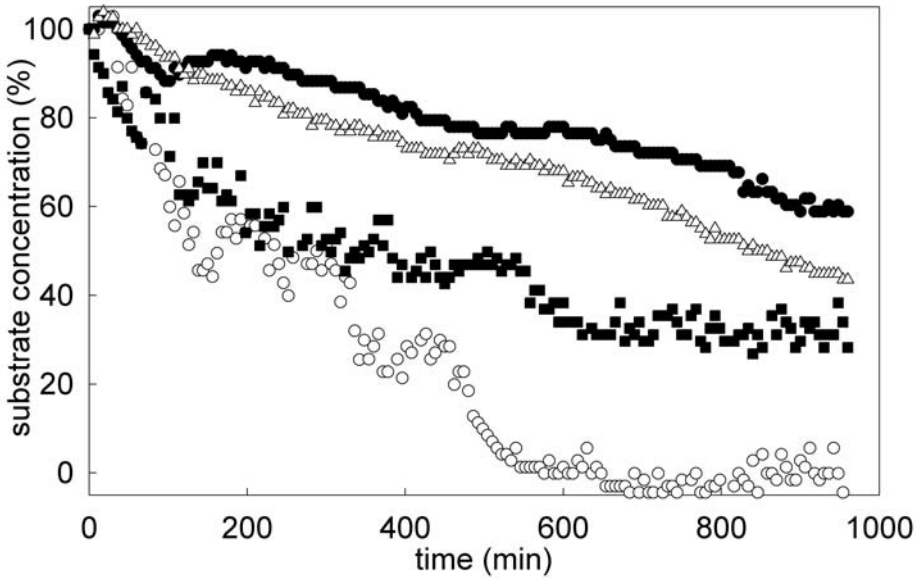


Figure 5.2. Effect of cross-linking time on CLEhC activity. Hydrolysis of racemic *p*-nitrostyrene oxide (pNSO) at an initial concentration of 150 μM was followed spectrophotometrically at 310 nm. CLEhCs were cross-linked with 5% v/v glutaraldehyde for either (○) 0.5 h, (■) 1 h or (△) 24 h. Conversion of pNSO by spontaneous hydrolysis only is shown for comparison (●).

When CLEhCs were stored in TEM buffer at 22 ^{o}C, no change in the protein concentration of the incubation solution (less than 1 μg ml^{-1}

measured by the Bradford method) could be detected over a period of at least 4 months indicating that CLEhCs were resistant to dissolution in buffer. This was confirmed by testing the buffer for epoxide hydrolase activity after incubation with CLEhCs. The pNSO conversion rate in this liquid was the same as that for spontaneous pNSO hydrolysis, again indicating that no active enzyme leaches from the cross-linked crystals.

Our results clearly show that the time of cross-linking is inversely related to the retained activity of the CLEhCs. Costes et al. (2001) indicated a trade-off between CLEC stability and activity after cross-linking, so that the degree of cross-linking should be the minimum possible to ensure sufficient stability without loss of activity. It would be useful to perform a more thorough screening of cross-linking conditions, to compare the effect on CLEC stability and activity of cross-linking carried out using a low concentration of cross-linking agent for a longer time with cross-linking carried out at higher cross-linking agent concentration for shorter times.

The optimal degree of cross-linking using glutaraldehyde is likely to vary for different enzymes depending on the number and location of lysine residues contained in the enzyme molecule. As already mentioned glutaraldehyde is often used as a cross linking agent for its ability to form cross-links spanning varying distances between lysine residues. The reactivity of the lysine side chains is determined by their spatial accessibility and their natural interactions with neighbouring side chains (O'Brien et al., 2001). Epoxide hydrolase contains 17 lysine residues which are located mainly on the outer surface of the enzyme molecule, exposed to solvent and far from the active site (PDB entry 1EHY). It seems unlikely that cross-links directly interfere with substrate conversion at the active site. More long ranging structural effects or lack of enzyme mobility at the active site, however, cannot be excluded. X-ray structural studies of the wild-type epoxide hydrolase unexpectedly found the Gln134 side chain positioned in the active sites which was explained as a consequence of crystal packing forces and the affinity of the active site residues for the amide group. Other structural effects could arise from crystallisation and the resulting packing forces and would need to be investigated by determining the X-ray structure of the Y215F mutant.

CLEhC activity

In order to reduce the amount of CLEhCs needed per experiment, an assay was developed for which reasonable changes in substrate concentration could be measured with reaction volumes of 200 µl. A typical progress curve for the hydrolysis of racemic pNSO is shown in Figure 5.3. The progress curve displays a biphasic shape with a change in turnover rate after 50% of the total substrate was converted. The second phase of conversion had a rate close to that of spontaneous hydrolysis indicating a

relatively high enantioselectivity, which is supported by the results reported below.

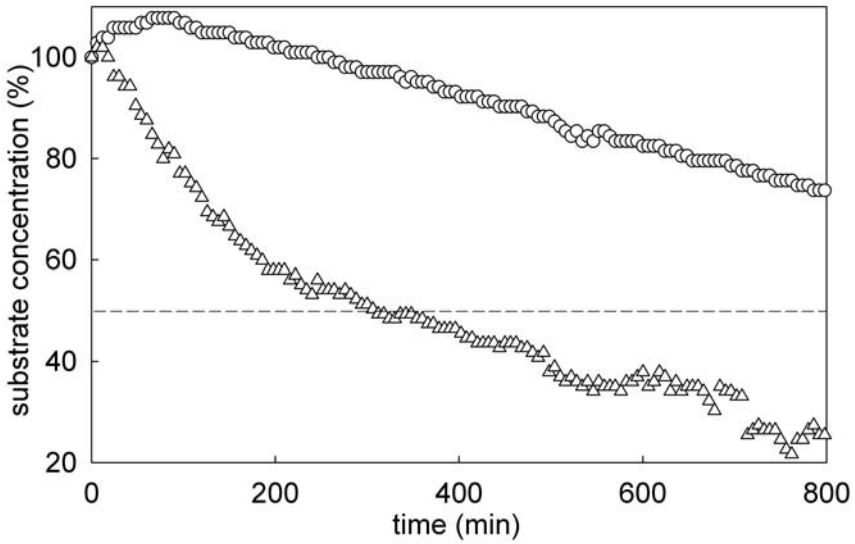


Figure 5.3. Typical conversion of racemic pNSO in CLEhC activity assays measured spectrophotometrically in the 96 wells-plate arrangement. Initial pNSO concentration was 150 μM in 200 μl TEM buffer. Both (△) CLEhC conversion of racemic pNSO and (○) spontaneous hydrolysis of pNSO are shown.

Good repeatability of the assay was essential for carrying out stability experiments, which were based on comparison of activity before and after incubation under certain conditions. The repeatability of the assay was tested for the conversion of 150 μM racemic pNSO using a 96-well plate reader spectrophotometer. Initial activities calculated for successive assays were comparable and ranged within a standard deviation of 20% from the average activity (Table 5.1).

Table 5.1. Reuse of CLEhCs for the conversion of racemic pNSO in successive batches.

Assay [a]	Initial rate, CLEhC [b] (dA_{310}/dt)	Initial rate, spont. [c] (dA_{310}/dt)
1	2.4×10^{-4}	4.6×10^{-5}
2	2.4×10^{-4}	4.4×10^{-5}
3	3.7×10^{-4}	4.3×10^{-5}
4	2.7×10^{-4}	3.8×10^{-5}
5	2.8×10^{-4}	5.0×10^{-5}

[a] A batch of CLEhCs was used for the conversion of 150 μM racemic pNSO in 200 μl TEM buffer, pH 7.5. Substrate conversion was followed by measuring the change in absorbance at 310 nm (A_{310}). Initial rates were calculated for no more than 20 % conversion. The same batch of CLEhCs was used for the five assays and between each assay the CLEhCs were washed 5 times with TEM buffer.
[b] Average initial enzymatic hydrolysis rate (dA_{310}/dt) = $2.8 \times 10^{-4} \pm 5.2 \times 10^{-5}$.
[c] Average initial spontaneous hydrolysis rate (dA_{310}/dt) = $4.4 \times 10^{-5} \pm 0.4 \times 10^{-5}$.

These results also show that the CLEhCs could be used at least 5 times without loss of activity, demonstrating good reusability which is desirable for large-scale application of CLEhCs.

The specific activity of the CLEhC preparation was estimated by measuring the initial rate of conversion of pNSO by the spectrophotometric method described above, using a known amount of enzyme in the CLEC form. The amount of enzyme used for each conversion was determined visually using a polarised light microscope by counting the individual CLEhCs and estimating their size. The concentration of enzyme in the crystals was estimated to be 8 mM (see below). Three separate reactions with different amounts of CLEhCs, visually observed to be approximately in the ratio 1:1.5:2.7, were carried out to obtain confidence in the calculated specific activity. For the soluble Y215F epoxide hydrolase mutant, the K_m values reported for the *(R)* and *(S)* enantiomers of pNSO were >0.8 and >1 mM respectively (Rink et al., 1999). Based on these values, the reactions were assumed to occur under first order conditions since the pNSO concentration in the assays was low (150 μM). This seems to be supported by the first order conversions observed using an even higher substrate concentration (0.5 mM) as shown in Fig. 5.7A. For these calculations the effects of mass transfer limitations were neglected. The observed rates of reaction were roughly proportional to the amount of enzyme present in each reaction volume. Based on the estimated amount of enzyme used for the conversions, an average apparent first order rate constant (k_{cat}/K_m) of 0.07 ± 0.01 $mM^{-1}s^{-1}$ was calculated. Since for the soluble epoxide hydrolase the specificity constant for the conversion of *(S)*-pNSO is two orders of magnitude lower than that of *(R)*-pNSO (Rink et al., 2000), the measured activity for CLEhCs was assumed to be mostly attributable to the conversion of *(R)*-pNSO. Therefore, the determined first order rate constant was taken as the specificity constant (k_{cat}^R/K_m^R) for *(R)*-pNSO. Based on this specificity constant, the activity of the CLEhC preparation was approximately 2% of that of the soluble Y215F epoxide hydrolase (Rink et al., 2000). The residual activity of CLECs has been reported to range from just a few percent less, to less than 1% of the activity of the soluble enzyme (Quiocho and Richards, 1966). The specific activity is affected by size of the crystals and the relative size of the substrate and the crystal channels (Margolin, 1996). If mass transfer limitations were present, then the specific activity determined by this method would be an underestimation of the intrinsic activity of the enzyme in the crystals. A change in the conformation of the enzyme in the CLEC form can also cause a decrease in specific activity as discussed below.

Enantioselectivity

The enantioselectivity of the CLEhCs was tested by performing kinetic resolutions of racemic pNSO and racemic styrene oxide (SO) (Fig. 5.4, and Fig. 5.5). The observed enantioselectivity was compared to that of the free enzyme in solution. The enantiomeric ratio, E, for the resolution of the racemic epoxides was calculated by fitting the degree of conversion, X, and the enantiomeric excess of the epoxide substrate, ee_s, to the well known formula (Chen et al., 1982) (Fig. 5.6, Table 5.2),

$$E = \frac{\ln\left[(1-X)(1-ee_S)\right]}{\ln\left[(1-X)(1+ee_S)\right]}. \qquad (5.1)$$

The apparent enantioselectivity of a kinetic resolution can be reduced as a result of the occurrence of unwanted side reactions. In this case, non-enantioselective spontaneous epoxide hydrolysis takes place during kinetic resolution. Because this side reaction causes a decrease in the yield of remaining enantiopure epoxide enantiomer, the E-ratio determined using the Chen formula is an underestimation of the intrinsic E-ratio for the resolution. The first order chemical hydrolysis rate constants for pNSO and SO were determined under the same reaction conditions as the enzyme reaction but in the absence of enzyme. The E-ratio corrected for spontaneous hydrolysis was determined by fitting the progress curves (Fig. 5.4A and 5.5A) to competitive Michaelis-Menten kinetics (Lutje Spelberg, 1998) taking into account chemical hydrolysis. The fitted kinetic constants were used to calculate the E-value by Eq. 5.2. The corrected E values, especially for styrene oxide, were still substantially lower than those found for the soluble enzyme (Table 5.2).

Table 5.2. Enantioselectivity of kinetic resolutions of styrene oxide and pNSO by both soluble and CLEC forms of the Y215F epoxide hydrolase mutant, and comparison to the wild-type epoxide hydrolase. E = enantiomeric ratio.

	Styrene oxide		pNSO	
	E-Chen [a]	E	E-Chen [a]	E
Wild-type epoxide hydrolase (soluble)	-	16.2 [d]	-	100 [e]
Soluble Y215F eh	18.7 ± 1.6 (26.6) [b]	-	69.4 ± 15 (87) [b]	-
CLEhC Y215F eh	2.6 ± 0.1 (2.9) [b]	3.4 ± 0.2 [c]	22.7 ± 1.0 (25.7) [b]	17.6 ± 2.8 [c]

[a] Calculated using the formula derived by Chen (1982), Eq. 5.1
[b] Corrected for spontaneous hydrolysis
[c] Calculated from conversion of the individual enantiomers with Eq. 5.2
[d] Lutje Spelberg et al. (1998)
[e] Rink et al. (1999).

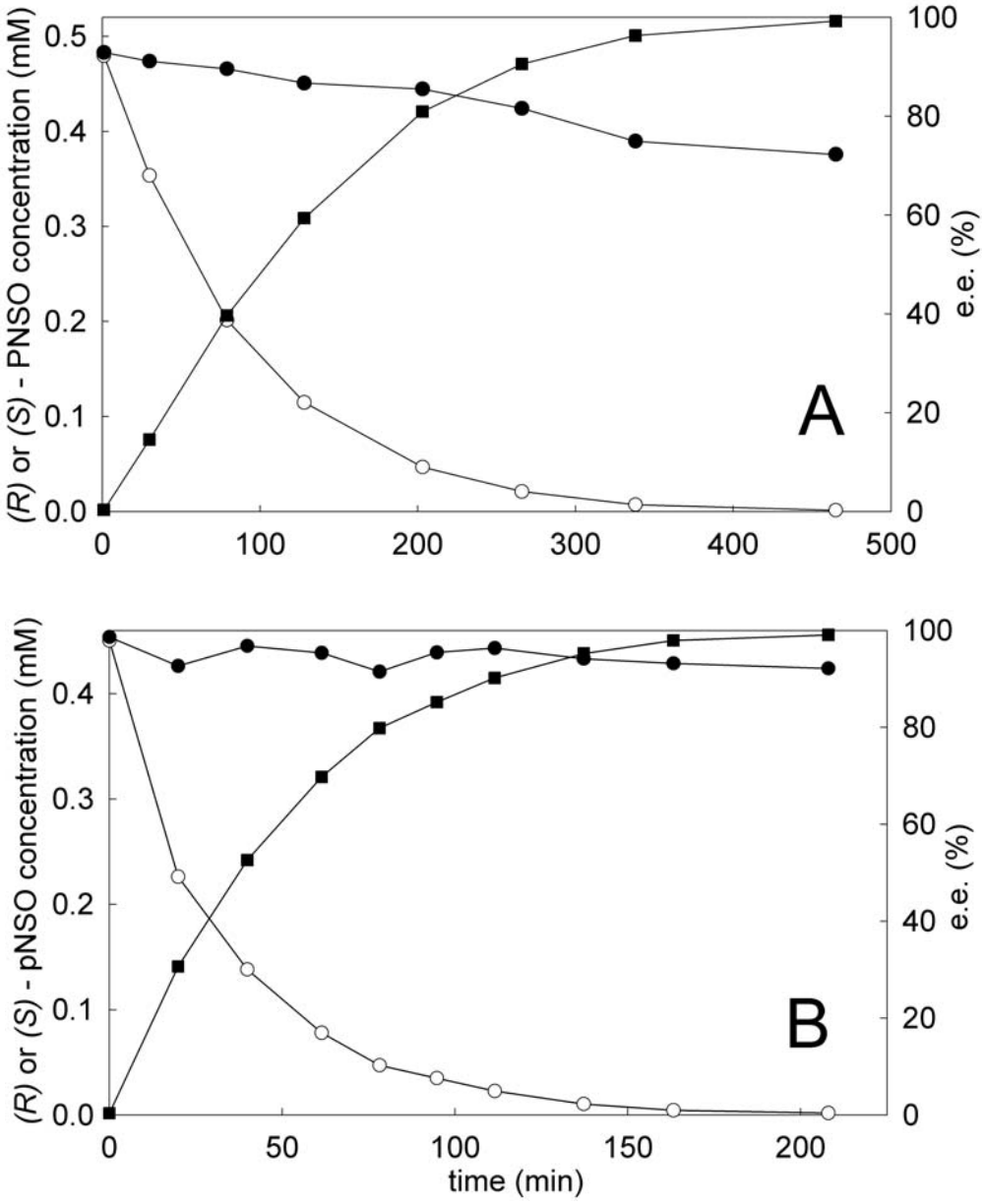


Figure 5.4. Kinetic resolution of 1 mM racemic pNSO by: **A**, Y215F CLEhCs, **B**, soluble Y215F epoxide hydrolase. (●) (*S*)-enantiomer, (○) (*R*)-enantiomer, (■) e.e.

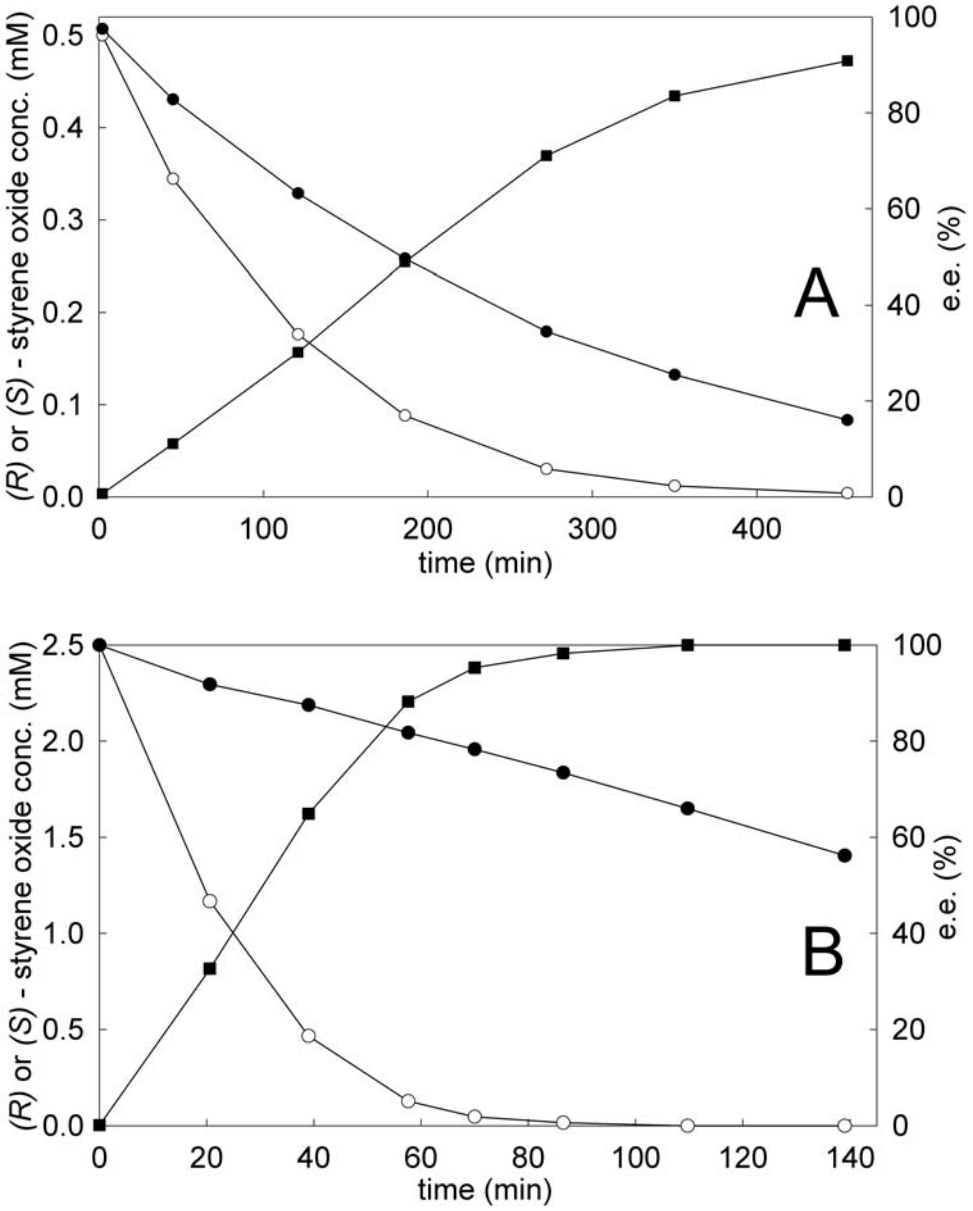


Figure 5.5. Kinetic resolution of: **A**, 1 mM racemic styrene oxide by Y215F CLEhCs, **B**, 5 mM racemic styrene oxide by soluble Y215F epoxide hydrolase. (●) (*S*)-enantiomer, (○) (*R*)-enantiomer, (■) e.e.

The enantioselectivity was also determined by comparing the rate of conversion of the individual enantiomers of the epoxides at the same CLEhC concentration. All single enantiomer conversions were carried out at 0.5 mM substrate concentration (Fig. 5.7). It was assumed that the conversions could be described by first order kinetics. This assumption was validated by the good first order exponential fit obtained for each data set, which also

suggested that the K_m values for the CLEC epoxide hydrolase may be higher than those found for the soluble enzyme (>0.8 mM)

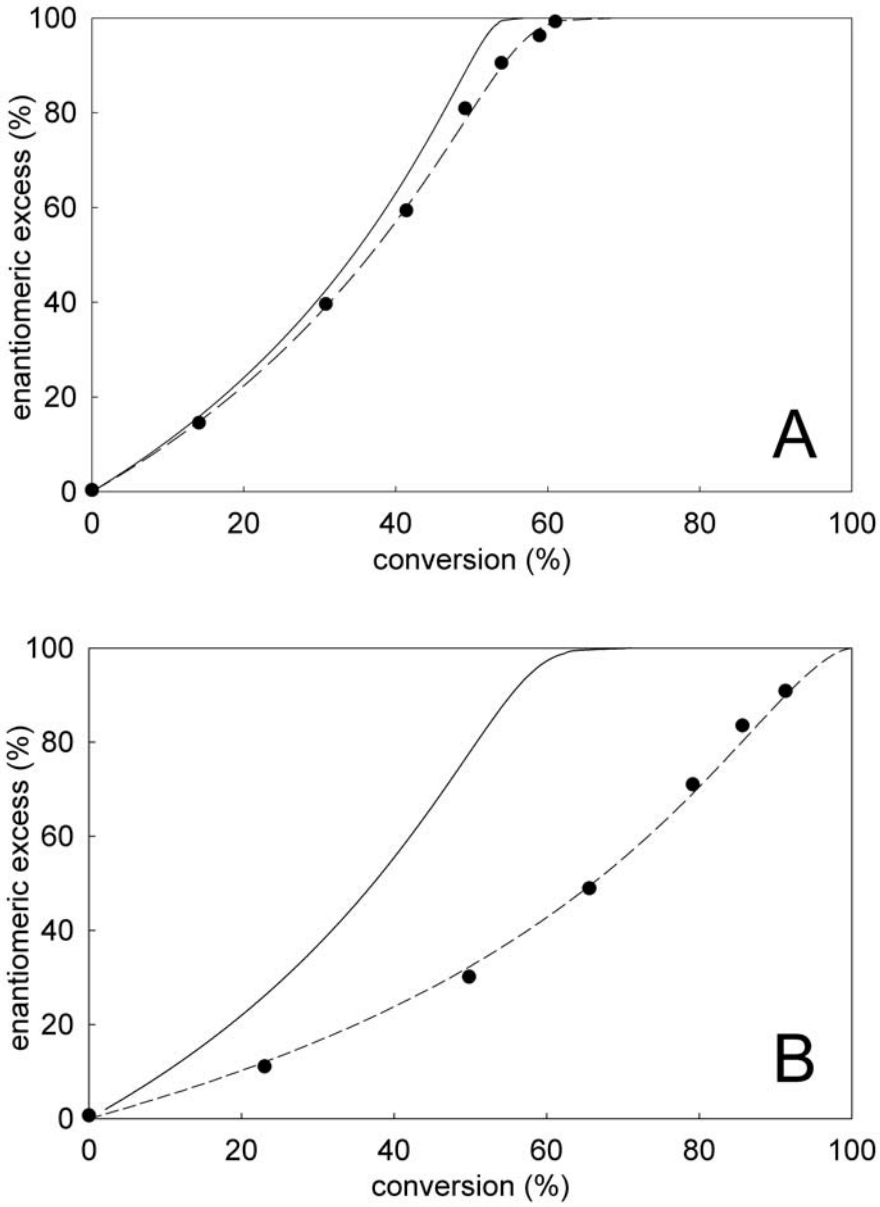


Figure 5.6. Enantioselectivity of epoxide kinetic resolutions carried out using soluble or CLEhC Y215F epoxide hydrolase. **A**: Resolution of 1 mM racemic pNSO. The lines are simulations for the fitted E-value of 23 (dashed) for resolution using CLEhCs, and 70 (solid) which is the enantioselectivity measured for the soluble enzyme. **B**: Resolution of 1 mM (using CLEhCs) or 5 mM (using soluble enzyme) racemic styrene oxide. The lines are simulations for the fitted E-value of 2.6 (dashed) for resolution using CLEhCs, and 19 (solid) which is the enantioselectivity measured for the soluble enzyme (Table 5.2).

(Rick et al., 1999). The E-values were calculated by fitting the progress curves (Fig. 5.7) to first order kinetics taking into account spontaneous hydrolysis. The enantiomeric ratio was then determined according to (Chen et al., 1982),

$$E = \frac{(k_{cat}/K_M)_R}{(k_{cat}/K_M)_S} \tag{5.2}$$

The values obtained were comparable to those determined from the kinetic resolution of the racemic mixtures. In both cases, the apparent enantiomeric ratio found using the CLEhCs was considerably lower than that found using the soluble enzyme (Table 5.2).

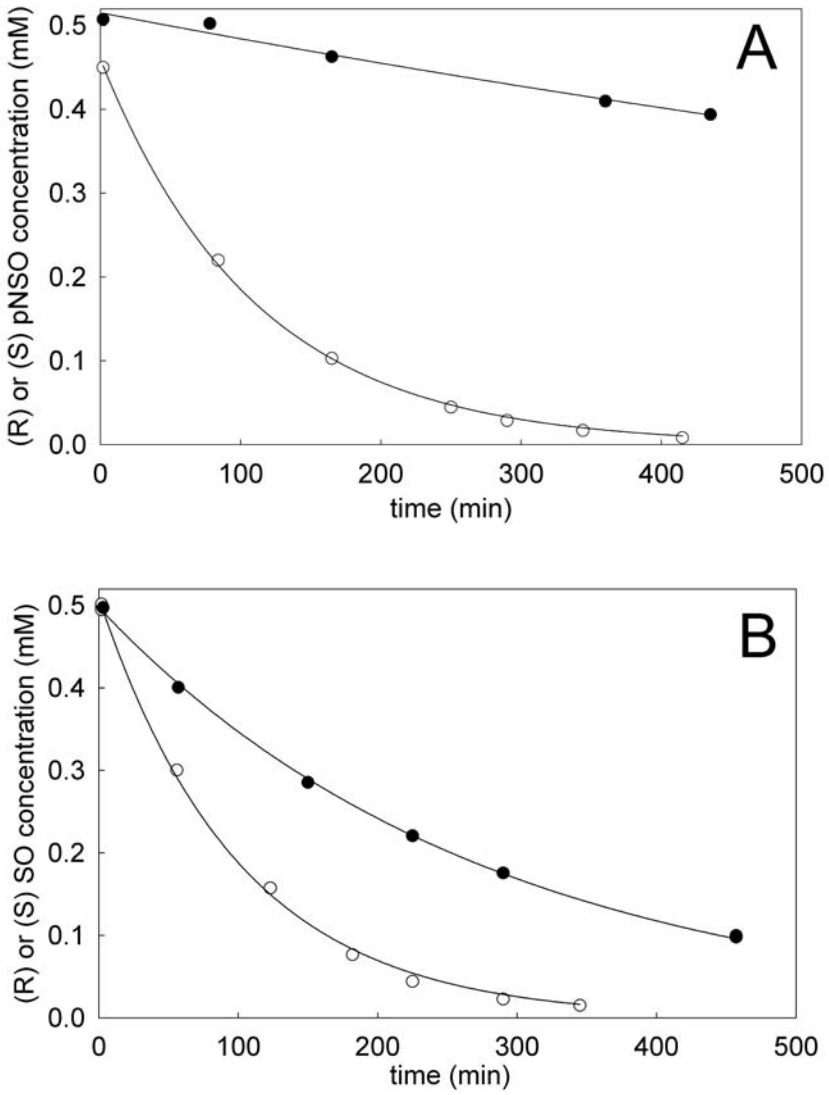


Figure 5.7. Conversion of epoxide pure enantiomers by CLEhCs carried out separately. **A**: Enzymatic hydrolysis of 0.5 mM (○) (*R*) and (●) (*S*)-pNSO. **B**: Enzymatic hydrolysis of 0.5 mM (○) (*R*) and (●) (*S*)-styrene oxide. Lines show fitted first-order decay curves.

Estimation of mass transfer limitations in CLEhCs

The apparent enantioselectivity of heterogeneous catalysts can be reduced as a result of mass transfer limitations (Barros et al., 2000, Litjens, et al., 2001, Matson and Lopez, 1990). Mass transfer limitations cause the formation of intra-particle concentration gradients for each enantiomer. The gradients are more severe for the faster reacting enantiomer, effectively reducing the availability of this enantiomer to the catalyst. The low apparent enantioselectivity calculated from the progress curves suggests that either mass transfer limitations in the CLEhC preparation are considerable, or that CLEhCs have different conversion kinetics compared to the soluble enzyme form.

For the following considerations, first order kinetics were assumed since the substrate concentrations used in the CLEhC conversions were low compared to the known K_m values for conversion of these epoxides with the soluble Y215F mutant. The good first-order fits of the progress curves for the conversion of the individual enantiomers by CLEhCs (Fig. 5.7) support this assumption. The rate of conversion of either enantiomer is described by,

$$\frac{dS}{dt} = -k_{obs}^{S} S + k_c S \,, \tag{5.3}$$

where, k_c is the first order rate constant for spontaneous chemical hydrolysis of epoxide. The observed first order rate constant for enzymatic conversion of the (*S*)-epoxide, k_{obs}^{S}, is given by,

$$k_{obs}^{S} = \frac{k_{cat}^{S}}{K_M^{S}} \eta_S [E], \tag{5.4}$$

and similarly, for the (*R*) - epoxide,

$$k_{obs}^{R} = \frac{k_{cat}^{R}}{K_M^{R}} \eta_R [E] \tag{5.5}$$

where, k_{cat} and K_m are the intrinsic Michaelis-Menten kinetic constants, [E] is the enzyme concentration and η is the effectiveness factor.

The effectiveness factors, η_S and η_R, represent the reduction in reaction rate due to mass transfer limitations. When there is no mass transfer limitation, then $\eta_S = \eta_R = 1$. By combining equations 5.2, 5.4 and 5.5, the following well-known relation between the intrinsic E value, E, and the effective (observed) E value, E_{eff}, can be derived (Litjens et al., 2001),

$$E_{eff} = E \frac{\eta_R}{\eta_S} \,. \tag{5.6}$$

If mass transfer limitations exist and the enzyme concentration is constant, then $\eta_R < \eta_S$ since R is the faster reacting enantiomer .

The effectiveness factor is a function of the Thiele modulus (Φ) which represents the relationship between the maximum rate of reaction and the

maximum rate of mass transfer according to (Tischer and Kasche, 1999; Westerterp et al., 1993),

$$\Phi = \frac{\delta}{2}\sqrt{\frac{k_{cat}[E]}{K_m D_{eff}}} \qquad (5.7)$$

where, δ is the thickness of the crystals, and D_{eff} is the effective diffusivity of the epoxide molecules in the CLEhC pores. For a first order reaction, and slab geometry (Alter et al., 1977; Westerterp et al., 1993),

$$\eta = \frac{\tanh\Phi}{\Phi}. \qquad (5.8)$$

From equations 5.6, 5.7 and 5.8 it follows that when $\Phi \to \infty$ (mass transfer limited system) $E_{eff} \to \sqrt{E}$ (Matson and Lopez, 1990).

The degree of mass transfer limitations which occur in the CLEhCs can be established by estimating the Thiele modulus for the faster reacting (*R*)-enantiomer, Φ_R. Generally, mass transfer limitations occur when $\Phi > 0.3$ (Westerterp et al., 1993), which from Eq. 5.8 corresponds to $\eta = 0.97$. Clearly, to estimate the Thiele modulus the enzyme concentration in the crystals is required. It proved difficult to determine [E] experimentally, but an estimate can be derived from knowledge of the crystalline structure of the wild-type epoxide hydrolase from X-ray crystallographic studies (Nardini et al., 1999). The study determined that a molecule of epoxide hydrolase has dimensions of 48 x 47 x 47 $Å^3$ in the crystal lattice and that the solvent content of the crystal was 52%. Accordingly, the enzyme concentration in the CLEhCs can be calculated as 8 μmol cm^{-3} (8 mM), which is in good agreement with other estimates made of enzyme concentration in the crystalline form (Alter et al., 1977). The maximum thickness, δ, of the CLEhCs was visually estimated to be 40 μm. The effective diffusivity was estimated based on the molecular diffusivity of styrene oxide in water (estimated as 5 x 10^{-6} cm^2 s^{-1}) and taking into account effects of steric hindrance based on the relative size of the CLEhC pore size (25 Å) (Perischetti et al., 1995) and the molecular diameter of styrene oxide, estimated as 7 Å. Accordingly, D_{eff} was calculated as 6 x 10^{-7} cm^2 s^{-1}. In the literature values of D_{eff} have been assumed to be approximately 50% of the free solution values, D_m (Alter et al., 1977; Spilburg et al., 1977; Tischer and Kasche, 1999), therefore our estimate of D_{eff} is a conservative one. Based on the estimated specificity constant k_{cat}^R / K_m^R of 0.07 $mM^{-1}s^{-1}$ it follows that $\Phi_R = 1.98$ and therefore $\eta_R = 0.48$. Assuming that, similar to the soluble enzyme, the specific activity for the conversion of *(S)*-pNSO for the CLEhCs is 2 orders of magnitude lower than for *(R)*-pNSO, $\eta_S = 0.99$. From Eq. 5.6, with E=87 determined for the soluble enzyme, the corresponding value of E_{eff} for pNSO would be 42. This value is still higher than that

observed experimentally (26) for the conversion of pNSO by CLEhCs (Table 5.2). This implies that the decrease in enantioselectivity cannot be fully explained by the occurrence of internal mass transfer limitations. The calculated values of η are approximations since all the parameters used for calculations (δ, k_{cat}^{R}/K_{m}^{R}, [E], D_{eff}) are estimates. For unchanged specific activity and for equal [E] and D_{eff}, mass transfer limitations would not exist if the width of the CLEhC was reduced by a factor of 10, to less than 4 μm.

From the calculations outlined above, we conclude that the decrease in enantioselectivity observed for kinetic resolutions using CLEhCs was at least partially caused by internal mass transfer limitations. External diffusion limitations, which arise due to a stagnant liquid layer around the CLECs can also reduce the observed rate of reaction, when $S < K_{m}^{S}$ or $R < K_{m}^{R}$, and enantioselectivity (Litjens et al., 2001). The stagnant liquid layer can be eliminated or reduced by sufficient mixing of the reaction medium so that external mass transfer limitations become negligible. It is assumed that external diffusion limitations were greatly reduced during the kinetic resolution experiments since these were well mixed. However, conversions carried out in the 96-well plates, which were shaken intermittently, may have suffered from such limitations even though increasing the frequency of agitation did not increase the rate of reaction (data not shown). The existence of external mass transfer limitations in the activity measurements carried out in the microwells would reduce the specific activity determined for the CLECs. This would result in an underestimation of the Thiele modulus, which was based on this measured value of specific activity, and would therefore also result in an underestimation of the decrease in enantioselectivity caused by the internal mass transfer limitations.

The possibility that the reduced enantioselectivity observed with the CLECs was at least partially caused by a change in the intrinsic conversion kinetics of the Y215F epoxide hydrolase mutant in the CLEC form cannot be ruled out. Enzymatic catalytic efficiency, k_{cat}, has often been found to be lower in the crystalline form, whereas both increases and decreases in the substrate binding affinity, K_m, have been reported (Alter et al., 1977). These changes have been attributed to minor structural changes that can occur in the enzyme active site upon cross-linking or crystallisation due to distortion of the crystal lattice (Quiocho and Richards, 1966), and due to restrictions in the mobility of the active site during conversion. It has been suggested that co-crystallisation with an inhibitor of the enzyme may help in retaining the correct alignment of catalytic residues in the active site during crystallisation (St. Clair et al., 2000), thereby retaining enzyme activity and enantioselectivity. It is possible that co-cross-linking with a competitive

inhibitor may also be used to the same effect. A small protective effect against loss of activity afforded by a competitive inhibitor during cross-linking of carboxypeptidase-A with glutaraldehyde was demonstrated by Quiocho and Richards (1966).

Product inhibition

The soluble form of the Y215F epoxide hydrolase mutant, which has been crystallised in this work, shows competitive inhibition by various diols to a similar extent as the wild-type enzyme (*Chapter 4*). To test further the suitability of the CLEhC preparation for large-scale use, the degree of inhibition by two diols, 1-phenyl-1,2-ethanediol (PED) and 3-phenoxy-1,2-propanediol (PPD), the products of styrene oxide and pNSO hydrolysis, respectively, was investigated. The competitive inhibition constants (K_i) for the soluble Y215F mutant are 31 mM and 49 mM for (*S*) and (*R*)-PED respectively, and 6 mM for racemic PPD (*Chapter 4*). Inhibition of the CLEhC preparation by these diols was tested by measuring the enzymatic activity for the conversion of racemic pNSO by CLEhCs incubated in diol solutions at various concentrations, compared to their activity in buffer. Results showed that no significant decrease in activity occurred when the CLEhCs were incubated in the diol solutions (Table 5.3). The decrease in activity expected in the presence of diol at the concentrations tested and with such low substrate concentration (150 μM) would be much larger than the 20% standard deviation in the activity measurements. This suggested that the inhibition constants for these diols were much higher for the CLEhCs than for the soluble enzyme, indicating reduced inhibition.

Table 5.3. Inhibition of CLEhCs by (*R*) or (*S*)-1-phenyl-1,2-ethanediol (PED) and 3-phenoxy-1,2-propanediol (PPD). The activity of CLEhC batches was measured in buffer and successively in a solution of the diols at various concentrations as shown. The change in activity is taken as the degree of inhibition caused by incubation of the CLEhCs at the particular diol concentration.

Diol	Diol conc. (mM)	Change %
(*R*)-PED	25	+ 11
	50	- 9
	75	+ 2
	100	+ 27
(*S*)-PED	15	+ 34
	30	+ 7
	52	+ 14
	75	+ 22
PPD	3.1	+ 3
	6.2	- 21
	9.3	+ 21
	12.4	+ 5

A reduced inhibition of the crystalline form of enzymes compared to the soluble forms has been reported in several different cases. For example, a 100-fold reduction in inhibition of carboxypeptidase-A crystals by β-phenylpropionic acid was found compared to the soluble enzyme (Quiocho and Richards, 1996). The lower apparent inhibition could be caused by a change in binding affinity of the diols in the CLEhCs active site, or by a reduced partitioning of the diol in the crystal lattice, which is expected to be a relatively hydrophobic environment compared to the aqueous buffer liquid. Indeed, the application of CLECs as chromatographic separation material is partly based on hydrophobic interactions between the intra-CLEC environment and the compounds to be separated (Pastinen et al., 2000). Size exclusion effects are generally also important but are unlikely to have much effect in this case since the size of the diol molecule is expected to be much smaller than typical crystal pore sizes. The reduced inhibition effect of diols on the CLEhCs would be an advantage in the use of CLEhCs for large-scale kinetic resolutions.

Stability

The thermostability of the CLEhC preparation was tested by comparing activity of a batch of CLEhCs before and after incubation at 60 ^{o}C for different incubation periods. At 60 ^{o}C the CLEhCs appeared to be more thermostable than the soluble enzyme (Fig. 5.8). After incubation at this temperature for 1 h the CLEhCs retained their activity completely, whereas the activity of the soluble enzyme decreased by 20% in the same time. The decrease in CLEhC activity seemed to level off after 100 min at 60% of the original activity, but after 22 h at 60 ^{o}C CLEhC activity had decreased by 70%. The decrease in CLEhC activity was correlated with an irreversible loss of crystal birefringence, which was observed visually under a polarised light microscope, although no change in the macroscopic shape of the crystals was observed. A similar loss of crystal birefringence has been observed in other studies concerned with thermostability of protein crystals (Jacob et al., 1998), and has been attributed to structural changes of the protein within the crystalline environment.

In large-scale processes, the use of high epoxide concentrations is desirable to obtain high reaction productivity. Cosolvents can be used to increase the concentration of epoxides in aqueous solutions since epoxides are often poorly water soluble. We investigated the stability of the CLEhCs in solutions of DMSO to determine whether CLEhCs could be used in the presence of cosolvents. When incubated with 50% v/v DMSO, the CLEhCs proved to be considerably more stable than the soluble enzyme form (Table 5.4). However, a decrease in CLEhC activity in time was observed, with 30% of the original activity left after 24 h, indicating that only a lower DMSO concentration would allow sustained enzyme activity and CLEhC reuse.

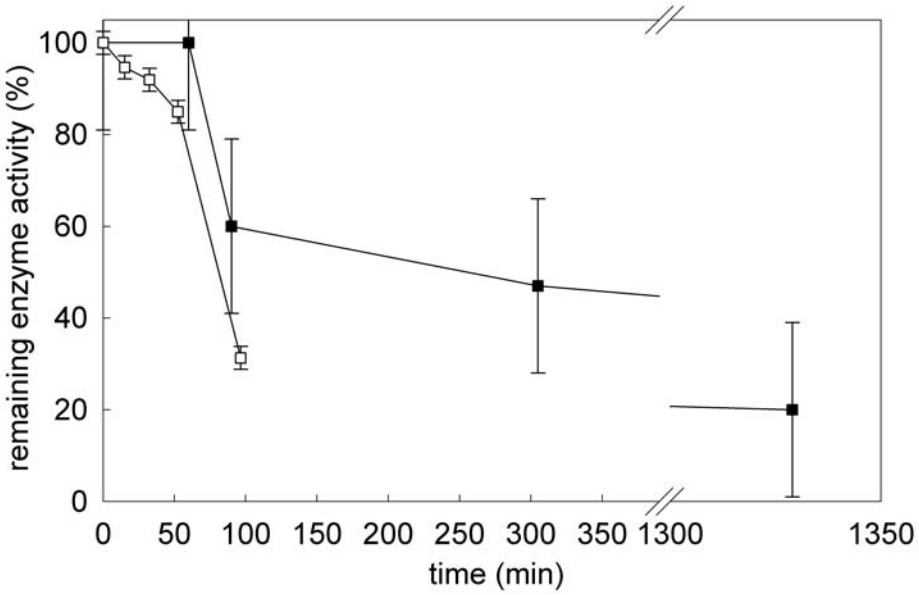


Figure 5.8. Thermostability of (□) soluble Y215F epoxide hydrolase and (■) Y215F CLEhCs. Enzyme preparations were incubated at 60 °C and remaining enzyme activity was measured at different points in time. Activities are given as a percentage of the initial activity.

The CLEhCs showed no loss of activity when incubated in an aqueous solution of 100 mM PPD over three hours. This is consistent with results found for the soluble form of the Y215F epoxide hydrolase, which was also stable in solutions containing high concentrations of this diol (*Chapter 4*).

Table 5.4. Stability of CLEhCs and soluble Y215F epoxide hydrolase in 50% DMSO. CLEhCs were incubated with DMSO at a concentration of 50% v/v. Activity was measured by the colorimetric assay before and after incubation for certain periods of time as given. The activity is reported as a percentage of the initial activity.

Enzyme form	Cumulative incubation time (min)	Remaining activity (%)
CLEhC	5	100
	70	67
	1440	33
Soluble Y215F	5	100
	42	49
	100	25

Soluble epoxide hydrolase thermostability

When the thermostability of the soluble Y215F epoxide hydrolase mutant was measured for comparison to the CLEhC preparation it was found that this mutant in the soluble form showed unexpectedly high stability at 60 ^{o}C. Earlier studies have shown that this active site mutant is more stable than the wild-type enzyme at high pH (Rink et al., 2000) and also when incubated in solutions of diols, such as (PED and PPD) at high concentration (*Chapter 4*). These findings prompted us to also compare the thermostability of this mutant with that of the wild-type enzyme. At 50 ^{o}C, the wild-type enzyme underwent a much more rapid decrease in activity than the mutant (Fig. 5.9). The activity of the wild-type enzyme decreased by 50% after 65 min incubation whereas the same reduction for the mutant occurred only after 1390 min. At 60 ^{o}C, however, the mutant activity decreased relatively rapidly and 50% activity loss occurred after approximately 80 min.

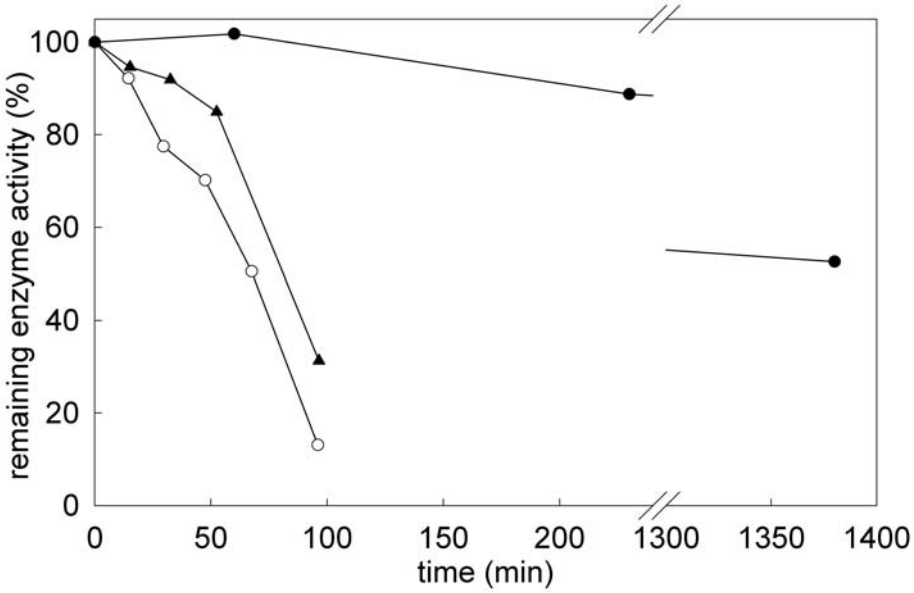


Figure 5.9. Comparison of the thermostability of wild-type and mutant (Y215F) epoxide hydrolases. Enzymes were incubated at the given temperatures and remaining enzyme activity was measured in time: (●) Y215F at 50 ^{o}C, (○) wt at 50 ^{o}C, (▲) Y215F at 60 ^{o}C. Activities are given as a percentage of the initial activity.

Conclusions

Crystals of the Y215F epoxide hydrolase mutant were formed by batch crystallisation. The crystals were cross-linked with glutaraldehyde to give CLEhCs which were resistant to dissolution in buffer. The CLEhCs were enantioselective and could be reused for repeated epoxide hydrolysis. The enantioselectivity of the CLEhCs was reduced compared to the soluble form

of the enzyme. The decrease in enantioselectivity was in part attributed to mass transfer limitations due to the thickness of the CLEhCs. Diol inhibition of CLEhCs was much reduced compared to the soluble form of the enzyme. This apparent reduction may have been partly caused by a lower partitioning of the diol molecules within the crystal pores. The CLEhCs also retained activity when incubated in diol solutions. The CLEhCs had a slightly higher thermostability than the soluble enzyme and showed higher stability in the presence of DMSO, making them attractive for large-scale application.

Chapter 6

Summary and concluding remarks

In this chapter the main findings of the work described in this thesis, aimed at the development of a process for the production of enantiomerically pure epoxides by enzymatic kinetic resolution, are summarised. The work was carried out using an epoxide hydrolase originally isolated from the organism *Agrobacterium radiobacter* AD1 (van den Wijngaard et al., 1989). The scale-up of this biocatalytic reaction is complicated by the poor solubility of epoxides in water, enzyme inhibition by the produced diol, and enzyme instability under reaction conditions (*Chapter 1*). Reaction productivity can be enhanced by using a liquid/liquid biphasic system so that high epoxide concentrations can be fed to the organic phase (*Chapter 2*). An n-octane/aqueous biphasic system was applied successfully to carry out kinetic resolutions with high epoxide concentrations, achieving high enantiomeric purity and reasonable yields. In such multiphase systems, the reduction of mass transfer limitations is crucial to obtain a high yield of enantiomerically pure epoxide. Preservation of enzyme activity during conversion is also a key factor in robust large-scale application of biocatalysts. Inactivation of epoxide hydrolase was found to be caused by direct contact with the organic liquid and by the presence of the diol product at high concentrations. An in depth study into the mechanism of enzyme inactivation at the liquid/liquid (L/L) interface was carried out (*Chapter 3*). Diol-induced enzyme inactivation was found to occur by a localised structural modification at the enzyme active site (*Chapter 4*). The active site mutant Tyr215Phe of epoxide hydrolase was resistant to inactivation by diols and since it also has a higher intrinsic enantioselectivity for conversion of some industrially interesting epoxides (Rink et al., 1999), it is an improved catalyst for large-scale application. The creation of cross-linked crystals of the Tyr215Phe epoxide hydrolase mutant (CLEhCs), which were active and enantioselective, is promising as it yielded a stable biocatalyst for application in a biphasic system. Future research should be aimed at the design of a three-phase system comprising the organic liquid that contains the substrate and the aqueous phase that contains CLEhCs of the epoxide hydrolase Tyr215Phe mutant.

Challenges in scale-up

The screening of organisms for epoxide hydrolase activity has been an area of considerable interest ever since the industrial potential for producing epoxides by biocatalytic methods was recognised (Archer, 1997; Archelas and Furstoss, 2001; Pedragosa-Moreau et al., 1993). The fruits of this work have been substantial and there are many examples of enzymes which have been isolated (Table 1.2), for some of which the gene has been cloned and overexpressed in preferred hosts such as *E. coli.* This includes the gene for the *A. radiobacter* epoxide hydrolase (Rink et al., 1997). These achievements have led to the facilitated production of these biocatalysts and

opened up the possibility of using them for preparative scale organic synthesis (Lutje Spelberg et al., 1998, 2002a). A review of the existing examples of preparative scale kinetic resolution that use epoxide hydrolases (*Chapter 1*) shows that the main difficulties in implementing these reactions on a preparative scale are the low solubility of the epoxide substrate and inhibition of the enzyme by the formed diol (Table 1.2). In many cases either cosolvents or immiscible solvents have been used to increase substrate concentrations. However, in general loss of enzyme activity during conversion seems problematic and it appears to be a crucial factor which has not been investigated in detail. Furthermore, in work described in the literature, the integration of the reaction step with downstream processing is usually overlooked. In particular the downstream separation of the unreacted epoxide substrate (desired product) and formed diol and also the recovery and potential reuse of the biocatalyst should be considered.

Research into chemo-catalytic routes for the production of enantiomerically pure epoxides, including by hydrolytic kinetic resolution (Table 1.3), has kept pace with research for biocatalytic methods (Schaus et al., 2002; Yoon and Jacobsen, 2003). High enantiomeric purity can be achieved in systems using very low concentrations of organic cosolvents with catalysts that are stable and can be recycled (Schaus et al., 2002). A full comparison between the chemical and biochemical routes for production of enantiopure epoxides is out of the scope of this thesis; however, it appears that the major advantage for the application of biocatalysis would come from being able to accommodate unusual substrate structures. In this respect, screening to find new enzymes with differing substrate specificity will remain important and makes the choice of epoxide used for screening purposes particularly important.

Multiphase systems

The productivity of kinetic resolutions of epoxides is limited due to the low aqueous solubility of epoxides. Multiphase systems can be employed to reduce this limitation, since the substrate can be fed at high concentration in the organic solvent. The kinetic resolution of styrene oxide in a liquid/liquid reaction system with octane as the organic solvent was evaluated (*Chapter 2*). Octane was chosen since it had the least effect on enzyme stability out of the solvents tested. This could be partly attributed to the low aqueous solubility of octane. However, the presence of a liquid/liquid interface still caused inactivation of epoxide hydrolase.

Due to the complex kinetics of the conversion of styrene oxide by this enzyme, whereby the conversion of the enantiomer that is converted first inhibits the conversion of the second enantiomer (Rink and Janssen, 1998), the relative rates of interphase mass transfer, enzymatic reaction, and enzyme inactivation determined the yield of enantiopure epoxide obtained

by kinetic resolution in the biphasic system (*Chapter 2*). Mathematical modelling of the reaction system was a powerful tool for determining the interaction between these three factors. The mass transfer parameters required in the mathematical model could be obtained by carrying out experiments with a stirred cell which has a well defined interfacial area. The interfacial area is a crucial parameter since it determines the rate of transfer of the substrate from the organic phase to the aqueous phase where the reaction takes place. Avoiding mass transfer limitations in the system was necessary in order to maintain a high observed enantioselectivity. A simple expression was developed which allowed calculation of the maximum allowed enzyme concentration in the aqueous phase for a certain interfacial area.

With further knowledge of the mechanisms of enzyme inactivation (described in *Chapters 3* and *4*), the model could be improved by including a more detailed description of the rate of enzyme inactivation as a function of mixing rate, the size of the interface present in the system, and the diol and epoxide concentrations in the organic phase. Validation of the model by measurement of remaining enzyme activity directly during conversion in the biphasic system would be required. This could be simply measured off-line by sampling the aqueous phase and carrying out assays with a colorimetric substrate (pNSO). With knowledge of the concentration of all species in the aqueous sample (epoxide and diol) and conversion kinetics (including inhibition) the remaining enzyme activity could be followed in time.

Stability of epoxide hydrolase

The perceived instability of enzymes is one of the causes for poor acceptance of biocatalysts compared to chemical catalysts. Enzyme stability should be assessed under reaction conditions i.e. in the presence of substrates and products, and organic solvents (Illanes et al., 1996). It is well known that enzymes can be inactivated in the presence of organic solvents. As mentioned above, octane was selected as the organic solvent for the biphasic system since it caused the least inactivation of epoxide hydrolase compared to the other solvents tested (*Chapter 2*). It was shown that the enzyme inactivation which occurred was due mainly to contact with organic/aqueous interface, and therefore the manner of interfacial inactivation was investigated further and is described below. The reduction in epoxide hydrolase activity observed in preparative kinetic resolutions which have been described in the literature has often been attributed to inhibition by the diol product (Choi et al., 2000; Genzel et al., 2001a; Tang et al., 2001), however the extent and reversibility of inhibition by diols was not investigated in detail. The effects of diols on epoxide hydrolase activity are described in *Chapter 4* and summarised below. Chemical modification of

epoxide hydrolase by highly reactive epoxides is also a potential cause of inactivation however this was not investigated in work carried out for this thesis.

Interfacial inactivation

Most investigations into enzyme adsorption and inactivation at liquid/liquid or solid/liquid interfaces are carried out in discontinuous systems from which information on adsorption kinetics and adsorption isotherms can be derived (Beverung et al., 1999; Roos and Walstra, 1996). However, in real reactor systems the processes of enzyme adsorption, inactivation and desorption from the interface must be considered together. The study conducted on the interfacial inactivation of epoxide hydrolase aimed to establish the effect of the interface on enzyme structure and activity, taking into account the nature of the biphasic systems encountered in real reactor systems (*Chapter 3*).

Interfacial inactivation studies were carried out with a stirred cell to control the interface present in the system. This allowed us to investigate the effect of increased interfacial shear on inactivation rate by increasing mixing rate without increasing the interfacial area. The size of the interfacial area determines the total amount of enzyme which is adsorbed to the interface at any point in time (Ghatorae et al., 1994), but the mixing rate determines the shear rate at the interface. Increased shear causes enzyme desorption from the interface and in turn also adsorption of enzyme from solution, thus determining the overall rate of enzyme inactivation by the interfacial mechanism. In emulsion systems the coalescence and break-up of solvent droplets contributes to the renewal of interfacial area in the system (Walstra, 1993).

The overall rate of enzyme interfacial inactivation was also affected by the aqueous phase enzyme concentration and the epoxide organic phase concentration (*Chapter 3*). It was hypothesised that a higher epoxide concentration in the organic phase could reduce the interfacial tension and increase the rate of droplet coalescence and break-up, thus forcing enzyme desorption and adsorption.

Circular dichroism analysis showed that the soluble epoxide hydrolase retains a native structure unless it inactivates by adsorption to the interface. Ultimately, the inactivation of epoxide hydrolase due to contact at the liquid/liquid interface results in aggregation and precipitation of the enzyme from solution. Sacrificial proteins, which bind preferentially to the interface, can be used to reduce the inactivation of enzyme, but both the sacrificial protein and the enzyme would eventually be inactivated so this may not be a practical solution at the large scale. The use of membranes to avoid contact between the enzyme and the organic phase would be an alternative reactor set-up (Choi et al., 2000).

Effects of diols on epoxide hydrolase activity

Vicinal diols are competitive inhibitors of epoxide hydrolase. We determined inhibition constants for some diols of varying structure for both the wild-type epoxide hydrolase and two active-site mutants (Y215F and Y152F) (*Chapter 4*). Vicinal diols with a large hydrophobic moiety had greater inhibitory potency. Inhibition constants were similar for the wild-type and mutant enzymes indicating that the determinants of diol binding in the active site were unchanged despite the single point mutation. Vicinal diols also caused slow time-dependent inactivation of wild-type epoxide hydrolase. The rate of inactivation increased with increasing diol concentration. Active site specificity of the inactivation was demonstrated by protection from inactivation by a strong competitive inhibitor. Furthermore, diols which had the higher inhibitory potency also had the greatest inactivating effect. Inactivation did not cause enzyme aggregation or precipitation from solution even though the inactivation was irreversible upon removal of diol. Circular dichroism spectroscopy showed that the secondary structure of epoxide hydrolase remained unaltered during inactivation but that changes occurred in the near-UV CD spectrum region assigned to tyrosine and tryptophan residues of the active site. The inactivation was attributed to a local structural modification which takes place at the active site specifically with vicinal diols. It was hypothesised that the inactivation mechanism might involve the formation of a covalent enzyme-diol complex through the formation of an ester bond, but attempts to identify the formation of such a complex by electrospray ionisation mass spectroscopy were unsuccessful. Assuming that the covalent complex formed between diol and enzyme is destroyed by acid catalysis prior to mass spectroscopic analysis, further efforts to identify this complex could make use of radiolabelled diols which would show whether diol is irreversibly bound to the enzyme even after removal of excess diol by dialysis. Further investigations should also determine the inactivating affect of pure enantiomers of the diols separately to gain further insight into the mechanism of inactivation since the different enantiomers could have different capabilities for forming a covalent adduct. The Y215F epoxide hydrolase mutant did not undergo inactivation during incubation with vicinal diols. Either the presence of both tyrosine residues in the active site is required for inactivation or the inactivation specifically involves Tyr215. Further tests to determining the stability of the Y152F mutant in contact with vicinal diols would distinguish between these two possibilities.

Epoxides are recognised as highly reactive compounds which makes them ideal intermediates in the synthesis of enantiomerically pure compounds since they can undergo ring opening with a wide variety of nucleophiles (Archer, 1997). Epoxides can react with amino groups of proteins through acid or base catalysed ring opening (Seidegaard and Depierre, 1983). Time-dependent inhibition of cysteine proteases by

peptidyl epoxides has been reported to occur by alkylation of the thiol of the active site cysteine residue (Albeck and Kliper, 2000). Further work should be carried out to investigate whether chemical modification of epoxide hydrolase resulting in inactivation can occur in the presence of epoxides. Incubation of inactive enzyme mutants, such as the Y215F+Y152F mutant (Rink et al., 2000), with epoxides in solution and monitoring of covalent modifications by LC-MS would be one strategy for carrying out such investigations.

Cross-linked crystals of epoxide hydrolase (CLEhCs)

The fact that enzymes in a crystalline form can retain activity has only recently been exploited for biocatalytic applications (Margolin and Navia, 2001). Cross-linking enzyme crystals makes them stable to dissolution and the high protein concentration environment within the crystal increases their stability under conditions of high temperature and exposure to organic solvents (Margolin, 1996; Noritomi et al., 1998). Further advantages recognised in the use of cross-linked crystals are facilitated enzyme recovery and recycle. In our hands, crosslinked epoxide hydrolase crystals of the Y215F mutant were created successfully and were resistant to dissolution in buffer (*Chapter 5*). The CLEhCs were active and enantioselective and could be used to carry out kinetic resolution of some epoxides to high enantiomeric excess and with a reasonable yield. The CLEhCs could be resued for at least five successive hydrolysis reactions without loss of activity. The observed enantioselectivity of the CLEhCs was lower than the intrinsic enantioselectivity of the soluble mutant enzyme. Calculations showed that some decrease in the observed enantioselectivity could have been caused by intra-CLEhC mass transfer limitations. Practical difficulties were encountered in the creation of large amounts of crystals because of poor repeatability of the crystallization step. The scale-up of this step is important to be able to collect more accurate data on the specific activity of the CLEhCs and thus to determine with greater accuracy the maximum crystal size permissible to avoid mass transfer limitations.

Product inhibition seemed to be greatly reduced for the CLEhC form compared to the soluble enzyme, providing a further advantage for application of CLEhCs in a large-scale system.

Wild-type vs. mutant (Tyr215Phe) epoxide hydrolase

Directed evolution has become an increasingly popular method for the optimisation of biocatalysts (Petrounia and Arnold, 2000). The work carried out in this thesis shows that site-directed mutagenesis can also be used successfully to improve biocatalyst performance. The active site mutant (Y215F) was shown to have higher enantioselectivity for the conversion of substituted styrene epoxides (Rink et al., 1999). Kinetic resolution by both enzymes yielded the enantiopure (*S*)-epoxide. The wild-type enzyme

demonstrated sequential kinetics and the rate of conversion of the second enantiomer increases after the first enantiomer is completely converted. This requires that the endpoint of the reaction be well-controlled to minimise loss of enantiopure epoxide. The mutant enzyme, however, showed more classical conversion kinetics (Rink et al., 2000) making this strict control less crucial. Both wild-type and mutant enzymes had broad pH optima from pH 6 to 10 (Rick et al., 2000). The active site mutant, however, was demonstrated to have a greater stability above pH 10 (Rink et al., 2000). This may not have direct advantages for large-scale application since the rate of spontaneous hydrolysis of epoxides (an undesired side reaction) is increased at extremes of pH. However, it may point to some general destabilising effect of the presence of the two tyrosine residues in the active site (Rink et al., 2000). We have determined that the mutant enzyme also has improved thermostability compared to the wild-type enzyme, although the stabilising mechanism is not known (*Chapter 5*). Furthermore, the Y215F mutant was not inactivated in the presence of vicinal diols which is a significant advantage over the use of the wild-type enzyme (*Chapter 4*).

The different conversion kinetics of the two enzymes have further consequences on bioprocess design. For the wild-type enzyme, product inhibition is characterised by competitive inhibition constants of 53 mM and 33 mM for the (*R*)-diol and the (*S*)-diol of styrene oxide, respectively (*Chapter 2*). However, since conversion of the styrene oxide is characterised by a high affinity of the substrate for the enzyme, with K_m values in the low micromolar range (Rink and Janssen, 1998), product inhibition only reduces the enzyme activity at diol concentrations greater than 1 M. For the Y215F mutant, inhibition constants are 49 and 31 mM for the (*R*)-diol and the (*S*)-diol of styrene oxide, respectively (*Chapter 4*). However, since the affinity for the separate enantiomers of styrene oxide is greatly reduced, with K_m^R=0.6 mM (Rink et al., 2000), the effect of product inhibition on the activity will be considerable at diol concentrations as low as 50 mM. This would make removal of diol from the aqueous phase a priority of process design in order to achieve the desired conversions.

Detailed characterisation of both the wild-type and mutant enzymes allowed identifying properties which were advantageous for large-scale application. It seems that in-depth characterisation of mutants obtained by directed evolution would be necessary before concluding that a particular mutant has improved properties for large-scale biocatalytic application. High-throughput screening of enzyme libraries aimed at obtaining biocatalysts with improved enantioselectivity is usually carried out by assessing enzyme enantioselectivity and activity (Reetz, 2001). However susceptibility to inhibition and stability of the biocatalyst under reacting conditions also determine the performance of the conversion. Screening of enzymes for stability, which is usually based on thermostability, may be

helpful, but this may not reflect the inactivation processes to which the enzyme is susceptible under reaction conditions (Illanes et al., 1996). The Y215F epoxide hydrolase mutant offers many advantages over the wild-type enzyme in terms of its application for large-scale kinetic resolution. It would be interesting to compare mutants obtained by directed evolution of the wild-type epoxide hydrolase with different evolutionary pressures (increased enantioselectivity, improved stability or reduced product inhibition) to the Y215F mutant.

Three phase solid/liquid/liquid system

The application of CLEhCs of the Y215F epoxide hydrolase mutant in a liquid/liquid biphasic system would be the ideal reaction system because:

- the cross-linked crystals of the Y215F mutant have good enantioselectivity;
- the inhibition by diols is greatly reduced compared to the soluble enzyme form;
- diols do not cause enzyme inactivation;
- the solid state of the enzyme would almost certainly eliminate or greatly reduce the occurrence of enzyme interfacial inactivation by contact with the separate octane phase and therefore high interfacial areas could be used to promote interphase mass transfer;
- the lower specific activity of the mutant epoxide hydrolase implies that the occurrence of intra-CLEhC mass transfer limitations is reduced compared to the wild-type enzyme. However, to completely avoid mass transfer limitations the maximum thickness of the CLEhCs would have to be controlled;
- recycling of the biocatalyst would be facilitated.

Ideally, such a reactor should be operated with the highest possible phase volume fraction of organic liquid in order to maximise the productivity. Factors which would determine the maximum organic phase volume fraction are the necessity to mix the two phases sufficiently and, since the diol does not partition to the octane phase, the maximum diol concentration permissible in the aqueous phase.

The use of a three phase reactor system would also be advantageous in terms of integration of the reaction step with downstream processing. The combination of an apolar liquid and polar environment results in a natural separation of the apolar epoxide (in the organic phase) and the more polar diol (in the aqueous phase) due to phase partitioning. The separation of the phases should be easily achievable by centrifugation and the recovery of epoxide by distillation. In general, the use of an apolar solvent which is less volatile than the product to be recovered is recommended to obtain a purer product and to reduce the cost of distillation (Mathys et al., 1998)

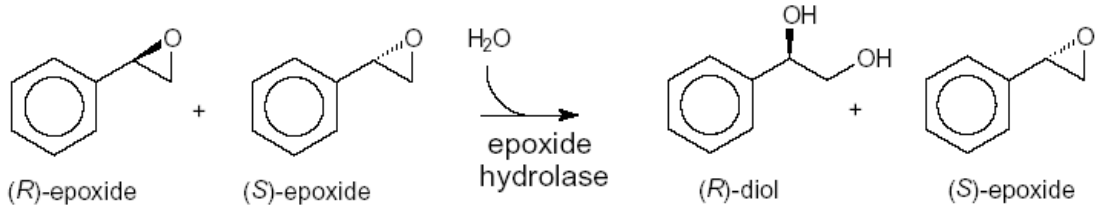
In summary, the limitations on the productivity of kinetic resolution of racemic epoxides can be greatly reduced by the use of multiphase reaction systems as detailed above. The application of CLECs of the mutant epoxide hydrolase (Tyr215Phe) provides an ideal catalyst for this purpose. The cheap and scalable production of the enzyme crystals remains a challenge, but is one which could likely be overcome with a readily available source of pure epoxide hydrolase. Ideally, the second phase should be composed solely of the epoxide substrate as a liquid or as a solid to eliminate the risk of contamination of the final product by an organic solvent. Therefore, the stability of CLEhCs or other immobilized forms of epoxide hydrolase should be tested in such systems.

Nederlandse samenvatting

Dit proefschrift beschrijft een onderzoek naar de toepasbaarheid van een epoxide hydrolase bij de productie van enantiomeerzuivere epoxiden door enzymatische kinetische resolutie. Epoxiden zijn reactieve verbindingen die goed aan verschillende andere moleculen gekoppeld kunnen worden. Door hun structuur en eigenschappen zijn het aantrekkelijke bouwstenen voor de bereiding van een scala aan farmaceutische producten. De werking van een farmaceutische verbinding is afhankelijk van zijn specifieke drie-dimensionale structuur. Veel farmaceutische verbindingen zijn asymmetrisch van structuur, dat betekent dat ze voorkomen in twee vormen (enantiomeren) die wel dezelfde chemische opbouw hebben maar toch een verschillende werking bezitten omdat hun drie-dimensionale structuur verschillend is: ze zijn elkaars spiegelbeeld. Met behulp van chemische methoden zijn asymmetrische verbindingen normaal gesproken eenvoudig te produceren als een 1:1 mengsel van de beide enantiomeren; dit noemt men een racemisch mengsel. Als een preparaat van een asymmetrische stof uit slechts één van de enantiomeren bestaat, wordt de stof enantiomeerzuiver genoemd. Het is tegenwoordig voor de meeste farmaceutische stoffen noodzakelijk dat ze in enantiomeer zuivere vorm geproduceerd en toegepast worden, met name om bijverschijnselen te voorkomen.

De chemische productie van asymmetrische verbindingen in enantiomeerzuivere vorm is vaak lastig. Eén van de methoden voor het in handen krijgen van enantiomeerzuivere stoffen is de scheiding van de enantiomeren die aanwezig zijn in een racemisch mengsel. Dit kan bijvoorbeeld gedaan worden door een enantioselectief enzym te gebruiken dat selectief reageert met slechts één enantiomeer van de asymmetriache verbinding. Deze methode wordt kinetische resolutie genoemd, waarbij op een bepaald moment in de reactie slechts de niet-reagerende enantiomeer in enantiomeer zuivere vorm overblijft. Een interessante groep van enzymen voor toepassing bij dergelijke kinetische resoluties zijn de epoxide hydrolasen.

Epoxide hydrolasen katalyseren de hydrolyse van epoxiden naar diolen. Deze enzymen komen voor in zoogdieren, schimmels, planten en bacteriën, waar ze een belangrijke functie hebben in het afbreken van giftige verbindingen. Het gebruik van epoxide hydrolasen voor de productie van enantiomeerzuivere epoxiden is pas gedurende de laatste jaren intensief onderzocht. Voor het werk dat in dit proefschrift beschreven wordt, is gebruik gemaakt van een epoxide hydrolase dat oorspronkelijk is geïsoleerd uit de bacterie Agrobacterium radiobacter AD1. In voorgaand onderzoek werd het gen voor dit enzym gekloneerd in de bacterie Escherichia coli, zodat het enzym in flinke hoeveelheden produceerd kan worden. Dit maakt het gebruik op grote schaal gemakkelijker. Gedetailleerd onderzoek naar het mechanisme van de enzymreactie en naar de enantioselectiviteit van het enzym voor de conversie van een scala van epoxiden is al beschreven voordat dit onderzoek begon (zie de proefschriften van M. Nardini, R. Rink en J.H. Lutje Spelberg).



Figuur 6.1. Conversie van en racemisch mengsel van styreen oxide m.b.v. epoxide hydrolase. Aleen de (*R*)-enantiomeer wordt omgezet in het corresponderende diol. De achterblijvende (*S*)-enantiomeer kan in zuivere vorm worden verkregen.

Voor een industriële toepassing van een kinetische resolutie die gebaseerd is op het gebruik van een epoxide hydrolase is een hoge productiviteit vereist. Dat wil zeggen dat relatief grote hoeveelheden enantiomeerzuiver epoxide geproduceerd kunnen worden in de kortst mogelijke tijd en met gebruikmaking van de kleinst mogelijke reactoren. Het doel van het werk in dit proefschrift was te onderzoeken hoe de productiviteit van de reactie te verhogen is zodat de toepasbaarheid op grote schaal binnen handbereik komt. De grootste uitdaging is dat de epoxide substraten slecht oplossen in water, terwijl dit het natuurlijke medium voor het functioneren van enzymen is. Een lage concentratie van substraat zou resulteren in de noodzaak grote reactoren te gebruiken, hetgeen economisch gezien zeer nadelig zou zijn. Een ander probleem ligt in het feit dat het diol dat geproduceerd wordt gedurende de reactie de enzymwerking onderdrukt, met nadelige gevolgen voor de katalytische efficiëntie en de productiviteit. Lage omzettingsnelheden leiden vaak tot lange reactietijden, hetgeen het proces oneconomisch maakt.

Een oplossing voor de beperkte oplosbaarheid is het gebruik van organische oplosmiddelen waarin het epoxide dat als substraat gebruikt wordt kan oplossen tot een hoge concentratie. Octaan kan bijvoorbeeld styreen-oxide oplossen tot een concentratie van meer dan 100 maal de oplosbaarheidslimiet in water. Tijdens het promotie-onderzoek is het gebruik van een organisch oplosmiddel getest door het uitvoeren van een kinetische resoluties van racemisch styreen-oxide in een vloeistof-vloeistof systeem bestaande uit water en octaan (Hoofdstuk 2). In zo'n systeem is het epoxide voornamelijk opgelost in de octaan-fase terwijl het enzym in de waterfase zit. Tijdens de conversie zal het epoxide gaandeweg diffunderen naar de waterfase, waar de enzymreactie plaatsvindt. We vonden dat in een dergelijk twee-fasen systeem een kinetische resolutie succesvol toegepastkan worden met epoxide-concentraties van tenminste 50 maal hoger dan in een systeem dat enkel uit een waterfase bestaat. Door de bijzondere kinetiek van de conversie van het substraat blijkt een zorgvuldige controle van de snelheid van stofoverdracht van het epoxide naar de waterfase nodig om hoge opbrengst van enantiomeerzuivere epoxide te behalen. Vooral de manier waarop de twee vloeistoffen worden gemengd en de eigenschappen van het grensvlak tussen de vloeistoffen moeten geoptimaliseerd worden om voldoende substraatoverdracht naar de waterfase te realiseren.

Verder onderzoek wees uit dat het twee-fasen vloeistof-vloeistof systeem weliswaar gunstig was voor het gebruik van hoge epoxide concentraties, maar dat het grensvlak tussen de twee vloeistoffasen nadelige gevolgen kon hebben op de enzymactiviteit. De belangrijkste parameters die het proces van grensvlak-inactivatie beïnvloedden warden onderzocht met gebruikmaking van een geroerd vat-reactor (stirred cell) waarin zowel een waterfase als een organische fase aanwezig zijn. Het mengen van de vloeistoffasen kan gevariëerd worden terwijl ook de grootte van het grensvlak tussen de twee fasen ingesteld kan worden (Hoofdstuk 3).De aanwezigheid van het octaan/water grensvlak resulteerde inderdaad in enzym-inactivatie. Deze zgn. interfacial inactivation treedt op bij het transport van enzymmoleculen naar de interface, tijdends het verblijf van het enzym aan de interface en/of op het moment van desorptie van enzyme vanaf de interface. De totale hoeveelheid enzym die inactief raakt per tijdseenheid is proportioneel aan de grootte van het grensvlak tussen de gebruikte oplosmiddellen in het systeem. Met name de snelheid waarmee geïnactiveerde enzymmoleculen van de interface desorberen bepaalt de netto snelheid van enzyminactivatie. Vrijkomend grensvlak kan namelijk opnieuw actief enzym aan zich binden en dit vervolgens inactiveren. Experimenten met geroerde vaten wezen uit dat de mate van enzyminactivatie stijgt met een toename van de menging in de waterfase, hetgeen verklaard kan worden uit een verhoogde desorptie van inactief enzyme tijdens roeren. Een vergelijking van de mate waarin grensvlakinactivatie optreedt in geroerde vaten en in een emulsiesysteem, wees uit dat de snelheid van inactivitie per oppervlakteeenheid van het grensvlak lager was in het emulsiesysteem. Analyse van de enzymeigenschappen met spectroscopische technieken toonde aan dat de grensvlakinactivatie een aggregatie van het enzym veroorzaakte en leidde tot precipitatie van het enzym, maar dat al het nog in de waterfase opgeloste enzym zijn activiteit en karakteristieke structuur behield.

De inhibitie van het epoxide hydrolase door verschillende diolen die als product kunnen voorkomen is beschreven in Hoofdstuk 4. Incubatie van epoxide hydrolase in oplossingen die hoge concentraties diol bevatten, bleek onomkeerbaar verlies van enzymactiviteit te veroorzaken, waarbij enzymactiviteit niet hersteld kon worden door verwijdering van het diol uit de oplossing. De remming en irreversibele inactivatie traden vooral op met vicinale diolen. Onderzoek met behulp van biofysische technieken zoals circulair dichroïsme spectroscopie toonde aan dat de inactivitatie geen grote veranderingen in de enzymstructuur teweeg bracht, maar wel gepaard ging met een lokale modificatie dicht bij het actieve centrum. Waarschijnlijk vindt er een covalente modificatie plaats waar de katalytische aminozuren bij betrokken zijn. De inhibitie en inactivitatie door diolen werden ook onderzocht met een gemuteerd epoxide hydrolase (Tyr215Phe), waarin een tyrosine in het katalytisch centrum die een rol speelt bij het openen van de epoxide ring, vervangen is. In voorgaand onderzoek bleek dat deze mutant, ten opzichte van het wild-type enzym, een verbetert enantioselectief gedrag vertoonde met

betrekking tot kinetische resoluties. Hoewel dit gemuteerde enzym in dezelfde mate als het wild-type enzym geremd werd door diolen, werd het niet irreversible geïnactiveerd. Daarom is het gemuteerde enzym meer geschikt voor industriële toepassingen dan het wild-type epoxide hydrolase.

Om de enzymstabiliteit in een vloeibaar-vloeibaar systeem te verhogen onderzochten we de mogelijkheid om kristallen van epoxide hydrolase te gebruiken om de kinetische resolutie reactie uit te voeren. Enzymkristallen kunnen chemisch zodanig gemodificeerd worden (crosslinking) dat ze niet meer kunnen oplossen. In kristalvorm kunnen ze eenvoudig uit de reactievloeistof gewonnen en hergebruikt worden. We maakten met succes cross-linked kristallen van de Tyr215Phe epoxide hydrolase mutant, die actief en enantioselectief waren en hergebruikt konden worden voor meerdere opeenvolgende kinetische resolutie reacties.De activiteit van de kristallen was slechts een paar procent van die van het oplosbare enzym en het controleren van de grootte van de kristallen bleek cruciaal te zijn voor het bereiken van een hoge enantioselectiviteit. Toch blijkt uit de behaalde resultaten dat de toepassing van cross-linked enzymkristallen van de epoxide hydrolase mutant in een tweefasen vloeistof-vloeistof systeem de voorkeur heeft boven opgelost enzym voor het behalen van een hoge productiviteit in de kinetische resolutie van racemische mengsels van asymmetrische epoxiden.

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